Molecular analysis of double-stranded RNA viruses in Agaricus bisporus and associated fungi

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MOLECULAR ANALYSIS OF DOUBLE-STRANDED RNA VIRUSES IN *AGARICUS BISPORUS* AND ASSOCIATED FUNGI

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IN THE NAME OF GOD

THE MOST COMPASSIONATE THE MOST MERCIFUL

To

My Parents

ABSTRACT

Mycoviruses have been associated with La France disease of the cultivated mushroom *Agaricus bisporus*, responsible for serious yield losses. *A. bisporus* is affected by different pathogens including fungi, such as *Verticillium fungicola* and *Trichoderma harzianum*. Studies of the evidence for the presence of double-stranded RNA (dsRNA) mycovirus in *A. bisporus* isolate V95 from the UK showing typical symptoms of La France disease with a decreased growth rate, included the presence of dsRNA segments and isometric virus particles of 25 nm and 34 nm diameter in virus preparations. The dsRNA segments in *A. bisporus* ranged in size from 0.78 to 3.6 kb. Two major polypeptides approximately 23 and 63 kDa were detected. RT-PCR and nucleotide sequence analysis of L1 (3391 bp) and L5 (2371 bp) dsRNA segments of an *A. bisporus* isolate V95 from the UK showed 95% and 98% nucleotide identity, and comparison of amino acid sequence showed 93% and 97% identity with the published sequence data for the L1 and L5 dsRNA of *A. bisporus* virus 1 isolate from The Netherlands.

Six geographically distinct isolates of *Verticillium* spp. were assessed for the presence of extrachromosomal nucleic acid elements. Two isolates were found to harbour dsRNA elements. Five dsRNA segments were found in *V. fungicola* isolate V7-3 which ranged from 1.1 to 2.4 kb. Only one dsRNA segment of 0.7 kb was detected in *V. psalliotae* isolate V 5-2. Isometric particles approximately 20 nm in diameter were purified from the mycelial tissue of isolate V7-3(+dsRNA). Initial analysis suggests that the viral capsid consists of one associated major protein with a molecular weight of ca. 36 kDa. Comparison with V7-3h (-dsRNA, heat treated) indicated that significantly reduced growth rate, dry weight , sporulation and virulence were associated with the presence of dsRNA. Complementary DNA clones from V7-3 dsRNA 1 contained inserts of approximately 1.7 kb which were partially sequenced and shown to contain conserved amino acid motifs typical of virus RNA-dependent RNA polymerases (RdRp).

Fifteen isolates of *T. harzianum* from five geographic locations were examined for the presence of dsRNA. Six of the isolates contained dsRNA elements and were assigned to two groups based on the similarity of banding patterns after agarose gel electrophoresis. Four dsRNA fragments were evident from *T. harzianum* isolates T7, KPNT, T32, Th1c and two dsRNA fragments from Th3c and A006022. Their sizes were approximately 1.65 to 2.0 kb. Electron microscopy of partially-purified preparations and ultrathin sections showed isometric VLPs of about 25 nm in diameter. Initial analysis suggests that the viral capsid consisted of two associated major polypeptides with molecular weight of ca. 55 kDa and 61 kDa. Comparison of isogenic isolate T7 (+dsRNA) and T7h (heat treated) indicated that significantly reduced dry weight and sporulation were associated with the presence of dsRNA. Partial nucleotide sequence was derived from dsRNA using a random priming RT-PCR protocol. This revealed that cDNA clones from T7 dsRNA-2 contained inserts of approximately 910 bp and dsRNA-3 contained inserts about 825 bp. The putative amino acid sequence encoded by the partial dsRNA-2 sequence revealed 46 % similarity with a hypothetical 69 kD protein of turnip yellow mosaic virus and dsRNA-3 has a 41% similarity with amphiphysin and guanine nucleotide-binding protein alpha-3 subunit (G-protein).

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ABBREVIATIONS

A	Adenine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMV	Avian Myeloblastosis Virus
ATP	Adenosine triphosphate
bp	Base pair
C	Cytosine
$CaCl_2$	Calcium chloride
cm	Centimetre
cDNA	Complementary deoxyribonucleic acid
CsCl	Caesium chloride
$CsSO_4$	Caesium sulphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EM	Electron microscopy
EtBr	Ethidium bromide
EtOH	Ethanol
G	Guanine
g	Gravity (acceleration due to gravity)
g	Grams
H_2O	Water
HCl	Hydrochloric acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
kbp	Kilobase pair (number of bases in thousands)

kg	Kilogram
M	Molar
mA	Milliamperes
ml	Millilitre
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_2$	Magnesium sulphate
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
NaAc	Sodium acetate
NaCl	Sodium Chloride
NA_2HPO_4	Disodium hydrogen phosphate
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometre
nM	Nanomolar
OD	Optical density (now called absorbance)
OD260	Optical density at 260 nm wavelength
^{32}P	Phosphorus-32
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rmp	Revolution per minute
rRNA	Ribosomal RNA
RT	Reverse transcriptase
S	Svedberg unit (sedimentation coefficient ; 1 S = 10^{-13} sec)

SDS	Sodium dodecyl sulphate.
ss	Single-stranded
T	Thymine
Taq	*Thermus aquaticus* polymerase
TBE	Tris-borate EDTA
TEMED	N,N,N′,N′- Tetramethyl ethylenedimine.
Tris	Tris[hydroxymethy] amino methane.
TE	Tris-EDTA
U	Uracil
uv	Ultraviolet.
v/v	Volume / volume
V	Volt
VLPs	Virus-like particles.
w/v	Weight/volume.

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CHAPTER 1

Introduction

CHAPTER 1 Introduction

1.1 GENERAL INTRODUCTION.

Double-stranded RNA viruses are known in all major groups of organisms, from fungi, protozoa , bacteria , algae to higher plants and animals. The first demonstration of dsRNA in nature was for reoviruses by Gomatos and Tamm (1963b). Six families of dsRNA viruses are presently recognized by the International Committee on Taxonomy of Viruses (Murphy, *et al.*,1995). They are differentiated on host, number of genome segments and capsid structure (single or double shelled). Members of the family *Reoviridae* (10-12 dsRNA genomic segments packaged in double shelled virions) infect vertebrates, invertebrates and plants. The Pseudomonas phage ϕ6 (family *Cystoviridae*) is the only known dsRNA virus that infects bacteria . Bacteriophage ϕ6 was isolated from bean straw infected with *Pseudomonas syringae* pv. *phaseolicola.* It contains a genome of three segments of double-stranded RNA packaged inside a procapsid which is covered by a shell of protein P8 and lipid-containing membrane containing additional viral proteins. (Mindich, *et al.*, 1988)

Members of the families *Birnaviridae* (hosts: vertebrates and invertebrates) and *Partitiviridae* (hosts: fungi and plants) have bipartite genome segments that are packaged together (birnaviruses) or separately (partitiviruses) in single shelled virions (Ghabrial, *et al.*, 1995b). The dsRNA viruses with undivided genomes (the family *Totiviridae*) package their monopartite genome single-shelled virions, hosts: fungi and parasitic protozoa (Ghabrial, *et al.*, 1995c). The sixth report of the ICTV lists a sixth family of dsRNA viruses, the family *Hypoviridae* (host : fungi), whose members lack conventional virions and their dsRNA molecules are enclosed in host-encoded vesicles (Hillman, *et al.*, 1995). Whether the genome of hypoviruses are dsRNA or ssRNA is still a matter of debate (Table 1.1: from The International Committee on Taxanomy of virus , Murphy, *et al.*, 1995).

Table 1.1 The dsRNA Viruses : The International Committee on Taxanomy of virus.

Family	Genus	Type Species	Host
Birnaviridae	*Aquabirnavirus*	Infectious pancreatic necrosis virus	Vertebrates
	Avibirnavirus	Infectious bursal disease virus	Vertebrates
	Entomobirnavirus	*Drosophila* X virus	Invertebrates
Cystoviridae	*Cystovirus*	*Pseudomonas* phage ϕ6	Bacteria
Hypoviridae	*Hypovirus*	*Cryphonectria* hypovirus 1-EP713	Fungi
Partitiviridae	*Partitivirus*	*Gaeumannomyces graminis* virus 019/6-A	Fungi
	Chrysovirus	*Penicillium chrysogenum* virus	Fungi
	Alphacryptovirus	White clover cryptic virus 1	Plant
	Betacryptovirus	White clover cryptic virus 2	Plant
Reoviridae	*Orthoreovirus*	Reovirus 3	Vertebrates
	Orbivirus	Bluetongue virus 1	Vertebrates
	Rotavirus	Simian rotavirus SA11	Vertebrates
	Coltivirus	Colorado tick fever virus	Vertebrates
	Aquareovirus	Goden shiner virus	Vertebrates
	Cypovirus	*Bombyx mori* cypovirus 1	Invertebrates
	Fijivirus	Fiji disease virus	Plant
	Phytoreovirus	Wound tumour virus	Plant
	Oryzavirus	Rice ragged stunt virus	Plant
Totiviridae	*Totivirus*	*Saccharomyces cerevisiae* virus L-A	Fungi
	Giardiavirus	*Giardia lamblia* virus	Protozoa
	Leishmaniavirus	*Leishmania* RNA virus 1-1	Protozoa

1.2 MYCOVIRUSES

1.2.1 Discovery of Mycovirus

The discovery of virus in fungi developed out of interest in an antiviral activity associated with two *Penicillium* molds, *P. stoloniferum* (Ellis and Kleinschmidt, 1967) and *P. funiculosum* (Banks, 1968), and from research on a disease of the cultivated mushroom, *Agaricus bisporus*. *A. bisporus* has been cultivated in Europe for about 300 years and in the U.S.A. for about a century. Hollings (1962) discovered the first fungal virus by extracting three types of virus like particles (VLPs) from the fruit bodies of diseased mushrooms in England. These studies predated the first report of dsRNA in a virus (Gomatos and Tamm, 1963 a).

It is now known that the active antiviral component associated with *P. funiculosum* and *P.stoloniferum* is dsRNA of viral origin (Lampson, *et al.*, 1967 and Kleinschmidt, *et al.*,1968) and it has been shown that dsRNA and the isometric particles copurify from diseased mushrooms of *A. bisporus* (Mario, *et al.*, 1976 ; Barton and Hollings, 1979).

The presence of dsRNA genetic elements in fungi is not uncommon. Typically, fungal viruses are isometric particles 25-50 nm in diameter, and possess dsRNA. DsRNA elements are found either packaged in protein capsids (mycovirus), or as unencapsidated dsRNA molecules. The dsRNA mycoviruses may have segmented or unsegmented genomes , whereas the unencapsidated dsRNA elements usually consist of multiple segments and are often associated with cell membranes (Nuss and Koltin 1990 ; Ghabrial, 1995 a). Most of these have genomes of dsRNA ,but genomes of single-stranded (ssRNA) and DNA have also been described (Buck,1986). Unlike most viruses of animals , bacteria and land plants, fungal viruses (mycoviruses) do not have an extracellular phase to their multiplication cycle and are transmitted only by intracellular routes. Like animal, bacterial and plant viruses, the genomes of many fungal viruses are enclosed in protein capsids, forming particles with morphologies

similar to those of viruses of other taxa (Buck, 1986). A main function of the capsid of animal, bacterial and plant viruses is to protect the virus genome when the virus is outside the cell and, in the case of animal and bacterial viruses, to bind to cell surface receptors to initiate uptake of the virion or virus nucleic acid into the cell. Mycovirus capsids do not need these functions, but play other essential functions, e.g. the capsid protein of a yeast virus has both a structural function in providing genome protection, and also a catalytic function in decapping host mRNA to facilitate translation of viral mRNA (Wickner, 1996). However, a significant number of mycovirus genomes do not have a protein capsid.

DsRNA has been associated with the presence of virus-like particles (VLPs) detected by electron microscopy in a number of fungal plant pathogens including *Cochliobolus victoriae* (Ghabrial, 1995) and *Gaeumannomyces graminis* (Buck,1986) and *Botrytis cinerea* (Howitt, *et al.*, 1995). In the saprotrophic *Agaricus bisporus*, such particles are associated with the debilitating 'La France' disease of mushroom beds (Goodin, *et al.*, 1992). Typically these mycoviruses possess segmented genomes, with the dsRNA segments being separately encapsidated into isometric particles. In other cases, the dsRNA elements apparently remain unencapsidated, and some of these are associated with lipid-rich cytoplasmic vesicles, e.g. *Cryphonectria parasitica* hypovirus (Fahima, *et al.*, 1993) and *Alternaria solani* (Zabalgogeazcoa, *et al.*, 1997) and *Sclerotinia sclerotium* (Boland, *et al.*, 1993). Some are located in the mitochondria, e.g. dsRNA element from *C. parasitica* (Polashack, *et al.*, 1994), *Ophiostoma novo-ulmi* (Rogers, *et al.*, 1987) and *Puccinia* spp. (Kim and Klassen., 1987).

These virus-like particles (VLPs) are usually cryptic, i.e their presence or absence does not cause any phenotypic effect. Nevertheless, in *Saccharomyces cerevisiae* (Wickner, 1996) and *Ustilago maydis* (Koltin, *et al.*, 1980), they possess a well-defined function, conferring killer activity to the host cell. A similar VLP, associated killer phenomenon has been described in *Phaffia rhodozyma* (Castillo and Cifuentes, 1994 and Pfeiffer, *et al.*, 1996).

1.2.2 Double-Stranded RNA Virus Replication and Packaging

The overall replication cycles for dsRNA viruses were clear almost 20 years ago, only recently with the development of molecular cloning techniques and *in vitro* transcription, replication, and packaging systems have detailed mechanisms for these processes come into view. Studies, particularly with the yeast L-A system, as well as the study of the roles of chromosomal genes in viral propagation, are especially well developed (Wickner, 1993).

Viral Replication Cycles

The dsRNA replication occurs in the cytoplasm for all dsRNA viruses that have been investigated. Transcription, defined as the synthesis of virus (+) strands from a dsRNA template, takes place within viral particles or core particles. One interesting exception is the dsRNA replicons that mitigate the virulence of the chestnut blight fungus, *C. parasitica*. These are found in membranous vesicles and lack a substantial protein coat (Dodds, 1980 b).

The new (+) strands are generally extruded from the viral particles. These (+) strands are then translated to make viral proteins. It is then the same (+)strands that are packaged to make new particles or subviral particles. Once the new particles or cores have formed, (-)strand synthesis on the (+)strand template (replication) completes the formation of new dsRNA. In systems where the virus is destined for export, addition of new layers of protein and/or membrane completes the virus reproduction cycle.

L-A is a 4.6 kb dsRNA virus that encodes its major coat protein (Gag) and its RNA polymerase, made as a Gag-Pol fusion protein by ribosomal frameshifting. L-A coat proteins can separately encapsidate and replicate the "satellite" dsRNAs that encode a secreted protein toxin (killer toxin). The cycles for the L-A virus of *S. cerevisiae*, and its satellite dsRNAs, are shown in Fig 1.1, as these are the focus of this review. A host cell (chromosomal) *KEX* gene is needed for killer expression, *MAK* genes for viral propagation (maintenance of killer), and *SKI* genes for repressing viral propagation (mutants are superkillers).

In Vitro Systems

Since all known dsRNA virus particles include a viral RNA-dependent RNA polymerase (RdRp), viral RNA synthesis is easily observed in purified or even relatively crude virus preparations. These activities are primarily (+)strand synthesis on the viral dsRNA template if mature viral particles are used. Newly formed particles, containing only (+)strands, can be isolated and carry out (-)strand synthesis. Because neither the template nor the enzyme can be altered in these reactions, they are of use mainly to study the products of the reactions and the requirements for and effects of small molecules. The finding that L-A viral particles exposed to low salt conditions lose their dsRNA but can now specifically bind and replicate added viral (+)strands or suitable artificial RNAs, and can transcribe added viral dsRNA, has allowed study of a number of these processes in detail (Fujimura and Wickner, 1988).

Transcription (= (+)Strand Synthesis)

Different segments of the multisegmented dsRNA viruses share a common sequence at the 5′ ends of their (+)strands, the starting point for transcription and therefore a presumed recognition site. The template-dependent *in vitro* transcription system described for L-A should provide a method to directly identify signals recognized by the transcriptase, but this remains to be done. The L-A transcription reaction requires an extract of (uninfected) host cells and a high concentration of polyethylene glycol. Transcription is conservative in mammalian reo and rotaviruses and in the L-A virus of yeast but semiconservative in the bacteriophage $\phi 6$ and the Aspergillus and Penicillium viruses (Buck, 1979). The difference is only whether the template (-)strand remains annealed to the new (+)strand (semiconservative) or repairs with the parental (+)strand (conservative).

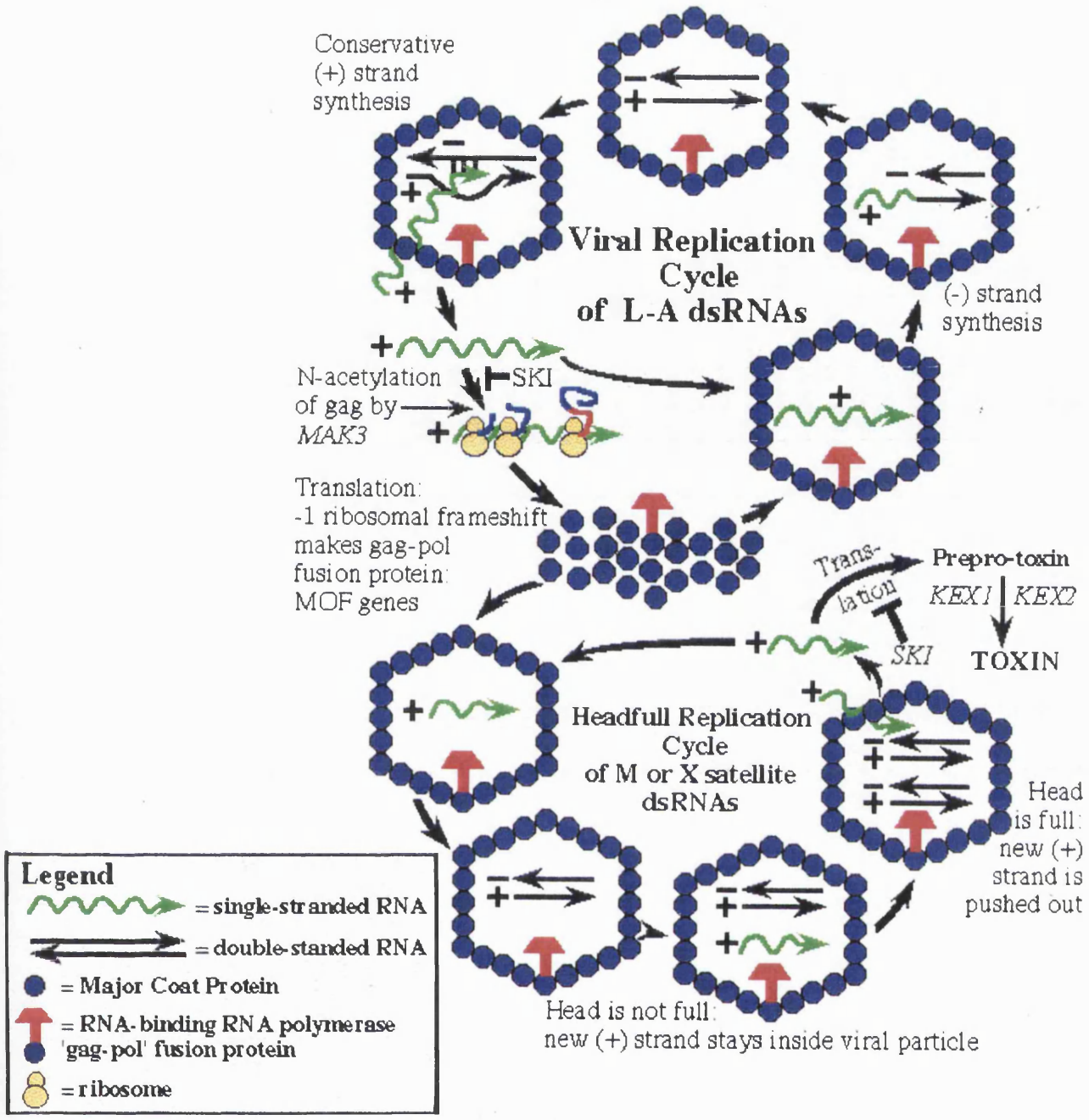


Fig 1.1 Replication cycle of the yeast L-A dsRNA (top), its deletion mutant X dsRNA, and the associated satellite M dsRNA (bottom). Both (+) and (-) strand RNA syntheses take place within the virions. (+)Strand synthesis occurs conservatively from the dsRNA template, and the newly synthesized (+)strand RNA is extruded from the particles. The released (+)strands serve as mRNA, as the species that is packaged to form progeny virions, and as the template for (-)strand RNA synthesis. Both M and X dsRNAs are less than half the size of L-A dsRNA, and depend on it for viral proteins. As in the replication cycle of L-A dsRNA, the (+)strand RNA of X or M dsRNAs are packaged and replicated. Because the virions are designed to accommodate one L-A dsRNA molecule per particle, the newly synthesized (+)strand RNAs are not extruded. As a result, the retained (+)strand is copied to form a second dsRNA molecule in the same particle, and the process continues until the particle is full, hence the term "headful replication". (Courtesy of Wickner, 1993)

Extrusion of Transcripts and the Headful Replication Model

New L-A or reovirus transcripts are all extruded from the particles. Such a possibility is suggested for rotavirus by their requirement for a hydrolyzable form of ATP for transcription. The satellite dsRNAs of L-A have genomes ranging from one-eighth to one-half the size of L-A itself. Particles that are full, having about 1 L-A of dsRNA, extrude all their new (+)strands from the particle, while those that are not often retain the new (+)strand and convert it to a second dsRNA molecule within the same particle as its parent. This can continue until there are as many as eight dsRNA molecules in one particle in the case of the small satellites of L-A that are about one-eighth the size of L-A. This suggests that extrusion of transcripts is simply a consequence of the heads being full and is driven by the energy of polymerization, not by a specific exportase. This is referred to as "headful replication" and probably applies to a number of single-segment fungal dsRNA viruses (Totiviruses) but perhaps not to the multisegmented dsRNA viruses.

Translation of (+)Strands

The (+)strand transcripts extruded from dsRNA viral particles serve as mRNA to make viral particle proteins. The (+)strands of L-A have two open reading frames, the 5′ *gag* ORF (680 residues) and the 3′ *pol* ORF (868 residues), that overlap by 130 base pairs. The *gag* ORF encodes the major coat protein, while *pol* has the consensus amino acid sequence patterns diagnostic of RdRp of (+)ss and dsRNA viruses. The pol ORF is expressed only as a Gag-Pol fusion protein (Fujimura and Wickner, 1988) formed by a -1 ribosomal frameshift event (Icho and Wickner, 1989), like that used by retroviruses (reviewed in Wickner, 1993).

Assembly and Packaging

The viral (+)strand is the species packaged to form new viral particles in all dsRNA viruses studied. Reovirus assembly and packaging are thought to occur in cytoplasmic inclusion bodies dense in virus protein. In the case of L-A, opened empty particles were found to bind specifically either L-A or M_1 viral (+) strands.

Expression of *gag* alone is sufficient to form morphologically normal (albeit empty) virus particles, indicating that the information for gross particle structure is independent of *pol.* However, the packaging of RNA having the viral packaging site requires the additional expression of the N-terminal fourth of the *pol* ORF (Fujimura, *et al.*, 1992). The Pol domain of the Gag-Pol fusion protein has ssRNA binding activity, and this suggests that Pol actually binds to the packaging site on the RNA. The Gag domain of the fusion protein presumably polymerizes with free Gag molecules to produce complete particles, each with a single viral (+)strand enclosed. The host factor needed for the *in vitro* (+) and (-)strand synthesis steps is presumably also packaged at this point.

That the ratio of Gag-Pol fusion protein to Gag protein is critical for viral propagation can be interpreted in terms of this model. If the Gag-Pol fusion protein is in excess, many more particles would be started, but there would not be sufficient Gag to complete them. A relative excess of Gag protein might result in the particles being closed before the *pol* domain had a chance to find a(+)strand to package.

Minus Strand Synthesis

L-A's (-)strand synthesis step has been studied in detail using the opened empty particles described above as the enzyme source and nature viral (+)strands or synthetic RNAs as templates (Fujimura and Wickner, 1988). The reaction is specific for viral (+) strands and requires an as yet uncharacterized "host factor", present in extracts of cells lacking L-A. The RNA signals required for template activity include the 3′-terminal region of the L-A (+) strand and an internal site 400 nucleotides from the 3′ end that largely overlaps with and may be identical to the packaging site. Within the essential 3′-terminal region of the L-A, the 3′end 4 bases are essential, as is immediately adjacent to the 3′-terminal region of the L- stem-loop. The sequence of the stem is not important, only that it be a stem, but the sequence of the loop is important. Remarkably, the 3′ end of M_1 that differs in sequence and structure from that of L-A can substitute for L-A's 3′ end.

The RNA polymerase probably binds first at the internal site, then loops around to find the 3′ end at which synthesis begins. It does not need to track along the RNA to find the 3′ end nor does the internal site stimulate polymerase activity by an allosteric effect (Wickner, 1993).

1.2.3 Transmission of dsRNA Mycovirus

Transmission of dsRNA mycovirus has been reviewed by Buck (1986) and Buck (1998).

Transmission through ascospores

Virus transmission into basidiospores, e.g. *A. bisporus* (Romaine, *et al.*, 1993), *Phaffia rhodozyma* (Pfeiffer, *et al.*, 1996) and *U. maydis*, and ascospores of yeast, e.g. *S. cerevisiae*, generally occurs with high efficiency. In contrast, virus transmission into ascospores of filamentous ascomycetes is often greatly restricted. In *Magnaporthe grisea*, only 10% of ascospore progenies contained dsRNA (Chun and Lee, 1997). Hypovirus-infected isolates of *C. parasitica* are female-sterile, and ascospore transmission of dsRNA in crosses between infected male parents and uninfected female parents is not known to occur (Nuss, 1992).

In a *C. parasitica* isolate carrying a mitochondrial dsRNA, the dsRNA was inherited maternally, as expected for mitochondrial inheritance. Although this isolate was hypovirulent, there was no evidence that the mitochondria were affected *per se* (Polashack and Hillman, 1994). However, in a d^2-infected isolate of *O. novo-ulmi* which carries ten mitochondrially located dsRNAs, only one out of 20 single ascospore isolates contained dsRNA ,and that isolate carried only one of the original ten dsRNA segments (Roger, *et al.*, 1986). This suggests preferential transmission of healthy, dsRNA-free, mitochondria into ascospore, since d^2-infected isolates probably contain a mixture of healthy and diseased mitochondria.

Chun and Lee, (1997) reported that 1.6 and 1.8 kb dsRNA have been found in the rice blast fungus, *Magnaporthe grisea* strain MG01. These dsRNA molecules were located in the cytoplasm of the fungal cells and maintained stable during vegetative growth. Three crosses between dsRNA free and dsRNA containing strains including a parental cross, sib-mating and black cross were made to follow the inheritance of dsRNA during sexual reproduction. Approximately 10% of ascospore progenies (11 out of 105) contained dsRNAs from all three crosses. This suggest that dsRNA of *M. grisea* was inherited at a low frequency and not in a Mendelian fashion.

Transmission through asexual spores

Virus transmission into asexual spores is generally efficient, although losses of some or all dsRNA segments can occur. In a d^2-infected isolate of *O. novo-ulmi*, 36 out of 45 single conidial isolates retained all ten of the parental dsRNA segments. The remaining nine isolates had lost between three and eight of the dsRNA segments. Similar losses occurred when the fungus multiplied as yeast-like cells in the elm xylem. Pronounced differences in the frequencies of loss of individual segments were noted (Roger, *et al.*, 1986). Transmission into conidia can be isolate-dependent. In hypovirus-infected *C. parasitica* (Melzer, *et al.*, 1997) and in virus-infected *Aspergillus* section *flavi* (Elias and Cotty, 1996), conidial inheritance of dsRNA varied from 0 to 100% depending on the isolate.

Transmission of dsRNA through conidia occurred in 15 out of 16 single-conidial isolates of culture 455 of *Pyricularia oryzae.* Among 16 single-conidial , 14 showed dsRNA banding identical to that of the parent isolate; one had no dsRNA, and one contained only one dsRNA of the parent. The partial transmission in one isolate (one dsRNA band of five normally found) suggests that the dsRNAs may be individually encapsidated or that there is a multiple-virus infection. Such complexes are common among mycoviruses (Bozarth, 1979). Also, because virus transmission through conidia was not 100 %, it is possible to obtain a virus-free isolate from an infected culture. This would be advantageous for transmission, biochemical and pathogenicity studies (Hunst, *et al.*, 1986).

Transmission by hyphal anastomosis

This method of transmission is generally limited to individuals in the same or closely related species. Virus transmission is efficient between individuals in the same vegetative compatibility (vc) group, but is restricted between individuals of different vc groups, because of post-fusion incompatibility, reactions which can lead to death of the fused cells. There are frequently several vc genes which control compatibility / incompatibility, and some of these may be multi-allelic. The strength of the compatibility reaction, and hence the degree of restriction of virus transmission, depends on the number of vegetative incompatibility (vic) gene differences, quantitative differences in the effects of individual vic genes and epistatic effects among vic genes (Brasier, 1986 ; Huber and Fulbright, 1994 ; Liu and Milgroom, 1996). One case of failure of virus transmission between compatible strains of *C. parasitica* was reported, but this probably was due to resistance to virus replication in the recipient rather than absence of dsRNA transmission (Polashack and Hillman, 1994).

Vegetative incompatibility in fungi is under genetic control and post-fusion incompatibility always results from differences in the vc genes. Each fungus has a set of genes responsible for vegetative incompatibility; for example, *C. parasitica* has six characterized loci, which gives a total of 64 vc types in this fungus (Cortesi and Milgroom, 1998). In most cases, for incompatibility to occur the alleles at all the loci must be identical, and differences in as little as one locus can lead to incompatibility between very closely related strains of the same species. Genes involved in vegetative incompatibility have been cloned from *Neurospora crassa* and *Podospora anserina*. In the former, these have been shown to be transcriptional regulators (Glass, *et al.*, 1990 ;Staben and Yanofsky, 1990) and in the latter, one gene has been shown to encode the α subunit of a heterotrimeric G protein (Loubradou, *et al.*, 1999).

The fungal population structure with regard to vc genes can have profound effects on virus spread. For example, in populations of *C. parasitica* in Italy, few vc groups have been found, and hypoviruses spread efficiently through the populations, leading to a natural biological control of the disease (Heiniger and Rigling, 1994).

In contrast, in North America, large numbers of vc groups have been found in *C. parasitica* populations, which could partially explain why a comparable hypovirus spread and natural biological control has not occurred. This problem can now potentially be circumvented by the availability of *C. parasitica* strains carrying cDNA hypovirus copies in their nuclear genomes with the potential to be disseminated into many vc groups by sexual crossing in the wild (Nuss, 1992).

Viruses causing deleterious effects have the potential to alter fungal population structures. In outbreaks of Dutch elm disease caused by *O. novo-ulmi*, populations at epidemic fronts are often clonal, consisting of a single vc group. This gives the opportunity for virus spread in the saprotrophic (bark) phase of the disease and, 50-90% of bark isolates at epidemic fronts have been found to be infected with disease-inducing (d-factor) mycoviruses (Brasier, 1986). However following an outbreak of the disease, there is a rapid increase in the numbers of vc groups in populations, paralleled by the virtual disappearance of d-factor mycovirus infection. It has been suggested that new vc groups could have arisen by the formation of rare hybrids between *O. ulmi* and *O. novo-ulmi* which are found in close proximity at new epidemic fronts of the more pathogenic *O. novo-ulmi.* Viruses could act as a selective agent for individuals in new vc groups which would be resistant to virus transmission and which would tend to outgrow infected individuals (Buck, 1998).

1.2.4 Effects of dsRNA Mycoviruses on Their Hosts.

Debilitation of the host

One of the best examples of viral-induced fungal debilitation is the d-factor of the Dutch elm disease fungus *O. novo-ulmi*. As with *R. solani*, many different-sized dsRNA genomes are found in *O. novo-ulmi* (Buck, 1998). Careful studies have correlated three particular elements with debilitation (Rogers, *et al.*, 1986). Although the presence of plasmids derived from the fungal mitochondrial (mt) DNA genome also correlates with the disease phenotype (Charter, *et al.*, 1993), this is thought to be the

result of dsRNA infection rather than the cause of it. The disease-causing dsRNA in *O. novo-ulmi* is found in the mitochondria and acts to decrease the levels of mitochondrial cytochrome c oxidase, leading to a respiratory deficiency in the fungus (Buck, 1998). The dsRNA in *O. novo-ulmi* has the potential to act as a biological control agent for Dutch elm disease, as the debilitated fungus is unable to infect elm trees. The spread of dsRNA in *O. novo-ulmi* depends on the structure of the fungal population (Sutherland and Brasier, 1997). If the population is clonal, there is endemic spread of dsRNA; if the population consists of a variety of vegetative incompatibility groups, the spread of the virus is restricted to strains of the same vegetative incompatibility group. However, the survival of a debilitating virus within a population of *O. novo-ulmi* suggests that it might spread more readily in a fungal populations than was previously thought, otherwise the selection processes would have eliminated the virus and its debilitated host (McCabe, *et al.*, 1999).

The role of protozoan viruses in host-parasite relationships remains largely unknown. In the case of *Trichomonas*, a correlation was found between the presence of the dsRNA virus and synthesis of a highly immunogenic protein on the trophozoite surface (Wang, *et al.*, 1987). Miller, *et al.*, (1988) have shown that the virus density in *Giardia* may reach $1x10^5$ per trophozoite, without affecting normal trophozoite appearance. They continue to adhere, although the growth rate is reduced to less than half. When the virus density reaches 2-5 $x10^5$ per trophozoite, however, parasites no longer adhere and growth apparently stops. In *Leishmania*, there is no evidence that the virus induces disease or affects growth characteristics of the protozoan (Weeks, *et al.*, 1992).

Killer viruses

The most studied phenotype associated with virus infection of fungi is the killer phenomenon, which is analogous to the bacteriocins in prokaryotes (Riley, 1998). Killer strains of fungi contain dsRNA genomes that encode a proteinaceous toxin to which the host fungus is immune, but which kills other strains of the same fungus that do not produce the toxin. Secretion of toxins could confer a selective advantage on organism in crowded environment. *S. cerevisiae* and other yeasts, such as

Hanseniaspora uvarum and *Zygosaccharomyces bailii,* have well characterized killer systems and several strains of the corn-smut agent, *U. maydis*, produce similar killer toxins (Park, *et al.,* 1996 a). Killer toxins confer a selective advantage to strains by allowing them to eliminate potential competitors occupying the same niche. Some toxins have a broad killing spectrum, a property that has potential for commercial exploitation (Schmitt, *et al.,* 1997).

The toxins secreted by killer strains of *U. maydis* are not related to their virulence or fitness as plant pathogens. The toxins have never been detected in infected plant tissues, and fungal strains lacking the toxin-encoding dsRNA segment are virulent (Nuss and Koltin, 1990). Because the toxins are not known to have deleterious effects on plant tissue, and because related species of *Ustilago* with various cereal hosts are sensitive to the toxins secreted by *U. maydis*, efforts are under way to produce transgenic plants that constitutively express the toxin as a means of biological control. The *U. maydis* killer toxins have been expressed in tobacco plants, resulting in high levels of secretion of a fully active toxin that is deleterious to plants infected with non-toxin-carrying *U. maydis* strains, while remaining harmless to toxin-expressing plants (Park, *et al.*, 1996 b). The efficiency of the killer toxins, together with the immunity conferred to the fungal cells that produce them, suggests that some use could be made of these organisms, or their toxins, for the biological control of plant diseases.

Hypovirulence in plant pathogenic fungi

Infection with dsRNA has been reported to reduce virulence in several virus-infected plant pathogenic fungi. The hypovirulence phenotype has been most carefully studied in the chestnut-blight fungus *C. parasitica.* Cryphonectria hypovirus 1 (CHV1) was first identified as a naturally occurring biological control agent for this very destructive disease of chestnut trees (Nuss, 1992). Fungal strains infected with CHV1 are unable to establish girdling cankers on chestnut trees and sporulate poorly. CHV1 does not cause general debilitation, but it does perturb fungal development and a variety of other effects, such as reduction of orange pigmentation, reduced conidiation, loss of female fertility, down-regulation of the production of oxalate, laccase (a polyphenol oxidase), cryparin (a cell surface hydrophobin),

putative mating-type A pheromones, cellulase (including a cloned cellobiohydrolase I), cutinase, protease (endothiapepsin), polygalacturonase and the α-subunit of a G protein (GTP-binding protein, CPG-1), and up-regulation of cAMP levels.

Several of these effects may be related to hypovirulence, e.g. down-regulation of cellulase is normally induced in the virulent pathogen-plant interaction and is likely to be required for plant cell wall hydrolysis. Down-regulation of G protein production appears to be central to the hypovirulence phenotype. G proteins are known to be involved in a number of signal transduction pathways in mammalian cells, and probably function by negatively regulating adenyl cyclase. Down-regulation of G protein production in *C. parasitica* is consistent with an increase in levels of cAMP and with the hypothesis that signal transduction pathways, normally induced in *C. parasitica* by its interaction with the chestnut tree host, are suppressed. Support for this hypothesis was obtained by the observations that transgenic co-suppression of CPG-1 in the absence of virus infection caused attenuation of virulence (Wang and Nuss, 1995), and targeted disruption of the *cpg*-1 gene resulted in phenotypic changes similar to, but more severe than, those caused by hypovirus infection (Gao and Nuss, 1996).

1.2.5 Satellite dsRNA and Defective dsRNA

Satellite RNAs, which are associated with many animal and plant ssRNA viruses, are RNAs which depend on a helper virus for their replication, but which are not required for the replication of their helper virus. Satellite RNAs are comprised largely of sequences which are distinct from those of their helper viruses (Mayo, *et al.*, 1995). Satellite RNAs may encode proteins or may be non-coding. The presence of satellite dsRNA genomes often explains the drastic differences in phenotype of these viruses. Satellite dsRNA are not physically linked to the genome of the helper virus, but they require their gene products for replication and encapsidation. The best known example is the satellite dsRNA associated with the *S. cerevisiae* L-A virus (ScV-L-A), which encodes a protein toxin responsible for the killer phenomenon (Wickner, 1986

and Schmitt and Neuhausen, 1994). Replication of the *S. cerevisiae* M1 satellite dsRNA is dependent on the presence of the L-A totivirus helper. The M1 toxin is expressed as a precursor protein which is broken down into its two subunits α and β by host proteases *Kex1p* and *Kex2p* (Wickner, 1996). M1 dsRNA can be eliminated (cured) from killer strains either by growth in the presence of sublethal concentrations of cycloheximide or by growth at elevated temperatures :39-40 °C (Wickner, 1986).

In the plant pathogen *U. maydis* , killer strains of the smut fungus secrete proteins which are toxic to sensitive strains of the same or closely related species. There are three distinct toxin specificities, KP1, KP4, and KP6, produced by different *U. maydis* strains (P1, P4, and P6 respectively); resistance to one toxin type does not confer resistance to the other two (Koltin, 1986). One of the M (medium, 1-1.6 kb) segments in each subtype encodes the killer toxin, and the KP4 and KP6 toxins are well characterized (Park, *et al.*, 1994 and Tao, *et al.*, 1990). The KP4 and KP6 toxins are encoded by the M2 dsRNA segments in each subtype. The KP4 toxin is a monomer of 11.1 kDa, but the KP6 toxin has two polypeptides, α (8.6 kDa) and β (9.1 kDa). The KP6 toxin is expressed as a pre-protoxin and proteolytically processed by the Kex2p protease (Park, *et al.*, 1994 ; Tao, *et al* .,1990).

However, the KPN4 toxin does not require *Kex2P* activity for its expression. Both the KP4 and KP6 toxins have been expressed from cDNA clones; both are expressed and secreted in active form in plant cells (Kinal, *et al.*, 1995 and Park, *et al.*, 1996 b). KP1 is the least understood of the three killer toxins, mainly because of its lower expression level (Park, *et al.*, 1996 a). Comparing the KP6 sequence with that of known killer toxins showed only similarity to the scorpion neurotoxins and cytotoxins. The KP6 α-polypeptide is of similar length to the neurotoxins (60-80 amino acids). Furthermore, the KP6 α-subunit, like the neurotoxins, has eight cysteines and is known to require intramolecular disulfide bridges for activity (Tao, *et al.*, 1990). KP6 α-polypeptide may also have a mode of action similar to that of the elapid cytotoxin , that is by forming ion channels. Despite lack of similarity in primary structure, the hydrophobicity profiles of the β-subunits of KP6 and the yeast K1 toxins are very similar, and those of KP1 and the yeast K2 toxins are almost identical (Tao, *et al.*,

1990). Whereas the β-subunit of K1 is linked to the α-subunit by disulfide bonds, it acts as a monomer in the case of KP1 and KP6. Furthermore, the β-subunit is glycosylated in KP1 and K2, but not in K1 or KP6.

Similarly , in *H. victoriae*, it has been proposed that satellite dsRNA might be responsible for the lytic disease of the fungus that results in hypovirulence (Ghabrial, 1994) . Satellite of partitiviruses from *Gaeumannomyces graminis* (Romanos, *et al.*, 1981) and *Atkinsonella hypoxylon* (Oh and Hillman, 1995), which encode protein of unknown function , and a noncoding satellite dsRNA of a *C. parasitica* hypovirus (Tartaglia, *et al.*, 1986) have been described.

Defective RNAs also depend on helper viruses for their replication but, unlike satellite RNAs, they are derived from their helper viruses, generally by internal deletions. Defective dsRNAs, derived by internal deletion of *C. parasitica* hypoviruses (Tartaglia, *et al.*, 1986 ; Shapira, *et al.*, 1991) and *S. cerevisiae* totivirus L-A (Esteban and Wickner, 1988), have been described. Deleted forms of the *S. cerevisiae* M1 satellite dsRNA (Lee, *et al.*, 1986) and *U. maydis* P1 M1 satellite dsRNA (Chang, *et al.*, 1988) have also been reported.

Both satellite and defective RNA can potentially interfere with the replication of their helper virus, probably by competing for replication factors. Both types of dsRNA are likely to be widespread in population of dsRNA mycoviruses and to contribute to the complexity of dsRNA profiles from individual fungi (Buck., 1998).

1.2.6 RNA-dependent RNA polymerase (RdRp)

Probably one of the first proteinaceous enzymes was RNA-dependent RNA polymerases (RdRp). Although there are several conserved motifs present in the RdRp of most positive and dsRNA viruses, the RdRps of the dsRNA viruses show no detectable sequence similarity outside the conserved motifs. There is now, however, a group of dsRNA viruses of lower eukaryotes whose RdRps are detectably similar.

The origin of this sequence similarity appears to be common descent from one or more noninfectious viruses of a progenitor cell, an origin that predates the differentiation of protozoans and fungi. The cause of this preservation of sequence appears to be constraints placed on the RdRp by the life-style of these viruses: the maintenance of a stable, persistent, noninfectious state (Bruenn, 1993).

Only one gene is common to all RNA viruses: a gene for an RdRp, or in some cases, an RNA-dependent DNA polymerase (reverse transcriptase). There is enough sequence conservation of several motifs within the RdRp that these have been used to identify this gene in many viruses for which no biochemical evidence is available to define this gene product. A number of attempts to define relationships among the RNA viruses have used sequence comparisons of the RdRp (Poch, *et al.*, 1989; Bruenn, 1991 and Koonin, 1991).They are thus the best criterion for molecular taxonomy of the RNA viruses. Although the secondary and tertiary structure of none of these is known, many primary structures have been deduced from genomic sequences. The RdRps of several positive strand viruses were first identified by sequence similarity to the known poliovirus polymerase by Kamer and Argos (1984).

One conserved motif, with a glycine-aspartic-aspartic (GDD) motif, was also noted in bacteriophage Qβ replicase and in the reverse transcriptases of the retroviruses. The availability of many more sequences of RNA viral genomes has enabled a number of investigators to identify several conserved motifs in the RdRps of positive strand, negative strand, and double-strand RNA viruses (Argos, 1988 ; Gorbalenya and Koonin, 1988 ; Habili and Symons, 1989 ; Icho and Wickner, 1989 ; Koonin, *et al.*, 1989 ; Pietras, *et al.*, 1989 ; and Poch, *et al.*, 1989) and in the reverse transcriptases (Argos, 1988 ; Poch, *et al.*, 1988). Most of the RdRp motifs have, in fact , been identified by primary sequence similarity with the known poliovirus RdRp, although there is genetic evidence identifying the RdRp coding sequences of the segmented positive strand and dsRNA viruses (Bruenn, 1980 ; Cornelissen, *et al.*, 1983; Mindich, *et al.*, 1988) and biochemical evidence identifying some of the dsRNA RdRp coding motifs as well (Fujimura and Wickner, 1988; Urakawa, *et al.*, 1989).

Among the dsRNA viruses, they are a very disparate group without easily detected sequence similarity over the entire length of their RdRps. For instance, the original publication describing the sequence of the RdRp encoding dsRNA of reovirus failed to identify it as encoding an RdRp (Wiener and Joklik ,1989), and the phi6 RdRp was reported as having no similarity to known RdRps (Mindich, *et al.*, 1988). However, both of these RdRps do have several of the conserved motifs characteristic of this enzyme (Morozov, 1989). A new group of dsRNA viruses from lower eukaryotes, however, demonstrates a very conserved RdRp. The peculiar (noninfectious) nature of these viruses leads to a possible explanation for this conservation in a common, ancient origin for RdRps.(Bruenn,1993).

The isometric dsRNA totiviruses infecting fungi and protozoa are unique among dsRNA viruses in that their genomes are undivided whereas the genomes of all other dsRNA viruses are segmented. The complete nucleotide sequences of eight totiviruses belonging to three genera in the family *Totiviridae* have been published. The genome organization and expression strategy of these viruses are similar; each virus contains two genes; the 5′ proximal encodes a coat protein (CP) and the 3′ proximal encodes an RdRp. Except for LRV2-1(Leishmania RNA Virus), the RdRp ORF overlaps the CP ORF and is in the -1 frame (Saccharomyces cerevisiae virus; ScV-L-A, ScV-La,: Helminthosporium victoriae 190S virus; Hv190SV and Giardia lanblia virus; GLV) or in the +1 frame (LRV1-1, LRV1-4, and Trichomonas vaginalis virus; TVV) with respect to the CP ORF. The RdRp ORF of LRV2-1 does not overlap the CP ORF (Ghabrial, 1998).

Sequence comparison analysis of the predicted amino acid sequences of totivirus RdRps indicated that they share significant sequence similarity and contain eight characteristic conserved motifs. This sequence similarity was common to the totiviruses that infect yeast and smut fungi as well as those infecting parasitic protozoa. The RdRp of Hv190SV, a recently characterized totivirus that infects a filamentous ascomycetous fungus, was also found to contain the same 8 conserved motifs (Huang and Ghabrial, 1996). The sequence similarity among the RdRps of these viruses infecting simple eukaryotes extends beyond the highly conserved 8 motifs. Of the 70 amino acid positions contained in these conserved motifs, the Hv190SV RdRp is

identical in 48, 47, 46, 40, 38 and 21 positions, respectively, to the RdRps of LRV1, TVV, Sc-L-A, UmVH1, ScV-La and GLV.

Bruenn (1993) questioned why the noninfectious dsRNA viruses of lower eukaryotes are much more closely related to each other than are the dsRNA viruses at large. The most likely explanation, according to Bruenn (1993), is that these viruses are monophyletic and that the progenitor was a noninfectious virus in a single cell type, and that cell type gave rise to both protozoa and fungi. Huang and Ghabrial (1996) finding that Hv190SV is more closely related to the leishmania viruses (LRV1 and LRV2) than to the yeast viruses (ScV-L-A and ScV-La) supports this hypothesis and suggests that Hv190SV and LRVs existed prior to the divergence of fungi and protozoa (Ghabrial, 1998).

1.2.7 Evolutionary Pathways of the dsRNA Mycoviruses

Theories of origin

Currently described viruses are very diverse and there is no compelling reason to suppose that all viruses arose in the same way. Not only does the nature of their genome vary (DNA or RNA), but the size of their genomes also varies enormously (Matthews, 1991). There are three different theories regarding the origin of viruses and these are reviewed in detail in Strauss, *et al.*, (1996) and Matthews (1991). One theory is that viruses evolved from autonomous, self-replicating, host cell molecules such as plasmids or transposons, by acquiring appropriate genes that code for packaging proteins. In prokaryotes there are strong parallels between some member of the bacteriophage families and bacterial plasmids. The phages contain large dsDNA genomes that, like some plasmids, can exist either in an integrated state in the host chromosome or as an autonomously replicating form. Their evolution as viruses is associated with their capacity to package their genome in virus particles which protect the genetic material while outside the host cell provide a mechanism for attachment to new host cells and DNA injection. The origin of the bacteriophage coat protein is not

known but it is interesting to note that they have some similarities in their structure and complex assembly patterns with bacterial self-assembly protein such as pilin and flagellin (Uhlin, *et al.*, 1985). Similarly in eukaryotes there are strong parallels between retrotransposons and the viruses that use reverse transcriptase.

Whether other RNA viruses arose in a similar manner or by *de novo* assembly from a polymerase core, is not known. Many types of uninfected plant cell contain RdRp (Strauss, *et al.*, 1996) and the replicaes function of many RNA viruses is an ancient evolutionary core feature that predates the cassette assembly process that generated the current RNA virus groups. With regard to the origin of the capsid proteins of the small icosahedral, positive strand RNA viruses of plants, insects and animals, it is interesting to note that the plant storage protein phaseolin, which has a packaging function, also has a domain containing the same eight-stranded, antiparallel β-barrel motif (Lawrence, *et al.*, 1990) found in the viral protein. Thus it is tempting to speculate that these isometric viruses arose first in plants and then subsequently evolved in insects before being transferred to mammals (Shukla, *et al.*, 1994).

A second theory is that some viruses arose by degeneration from primitive cells in a manner similar to that proposed for the evolution of cellular organelles such as mitochondria and chloroplasts from bacteria (Strauss, *et al.*, 1996). This process would entail: (i) the loss of a bounding membrane that separates the replication of the primitive parasitic cell from the host cell, thus prohibiting binary fission which is characteristic of cell division; and (ii) the use of host cell protein-synthesizing and metabolic machinery (Matthews, 1991). There are some problems with this type of explanation for the origin of all viruses (Strauss, *et al.*, 1996) but it has been suggested to be a possible mechanism by which the poxviruses arose (Fenner, 1979). Their very large genome size, their complex structure, the presence of many enzymes within the virus particle, and their ability to replicate in the cytoplasm independently of host nuclear functions suggest that these large, enveloped DNA viruses may have arisen from simple cellular parasites (Matthews, 1991).

A third theory is that some RNA viruses are descendants of prebiotic RNA polymers. RNA molecules can carry out nucleolytic cleavage, self-splicing reactions, ligations and even polymerization in a template-dependent fashion (Strauss, *et al.*, 1996 ; Matthews, 1991). In addition the tRNA structures found at the 3′ end of some RNA viruses have been suggested to represent molecular fossils of the original RNA world which tagged genomic RNA for the initiation of replication and functioned as primitive telomeres to prevent loss of terminal nucleotides during the replication process (Weiner and Maizels, 1987). This theory suggests that some RNA viruses might have evolved from the prebiotic RNA world and parasitized the earliest cells.

A fourth possibility is that some viruses evolved from viroids or virusoids although it is equally possible that these small RNAs, rather than being progenitors of viruses, are recent degenerative products of the more complex self-replication systems (Strauss, *et al.*, 1996). Neither code for any protein.

Totiviruses and Partitiviruses

Three theories are usually advanced when the origin of these viruses is considered. These theories entail viruses originating from either degenerate forms of intracellular parasites, self-replicating cellular mRNA, or from a prebiotic self-replicating RNA molecules (Koonin and Dolja, 1993). The theory of self-replicating cellular mRNA as the origin of totiviruses is attractive because of their apparent ancient origin, simplicity of genomes, close relationship among the RdRps and ability to use host proteins efficiently. A cellular mRNA (e.g. the mRNA encoding a DNA-dependent RNA polymerase) might have acquired the ability to replicate itself by obtaining an origin of replication. Provided this self-replicating mRNA could acquire a gene for a coat protein, then a very simple (+) strand RNA virus would be generated. Replication of (+) strand RNA involves the synthesis of a (-) strand RNA intermediate, and it is possible that dsRNA viruses may have arisen from (+) strand RNA viruses. Alternatively, cellular translation control mechanisms involving the production of antisense RNA, could also lead to the generation of dsRNA (Fig. 1.2 pathway 1).

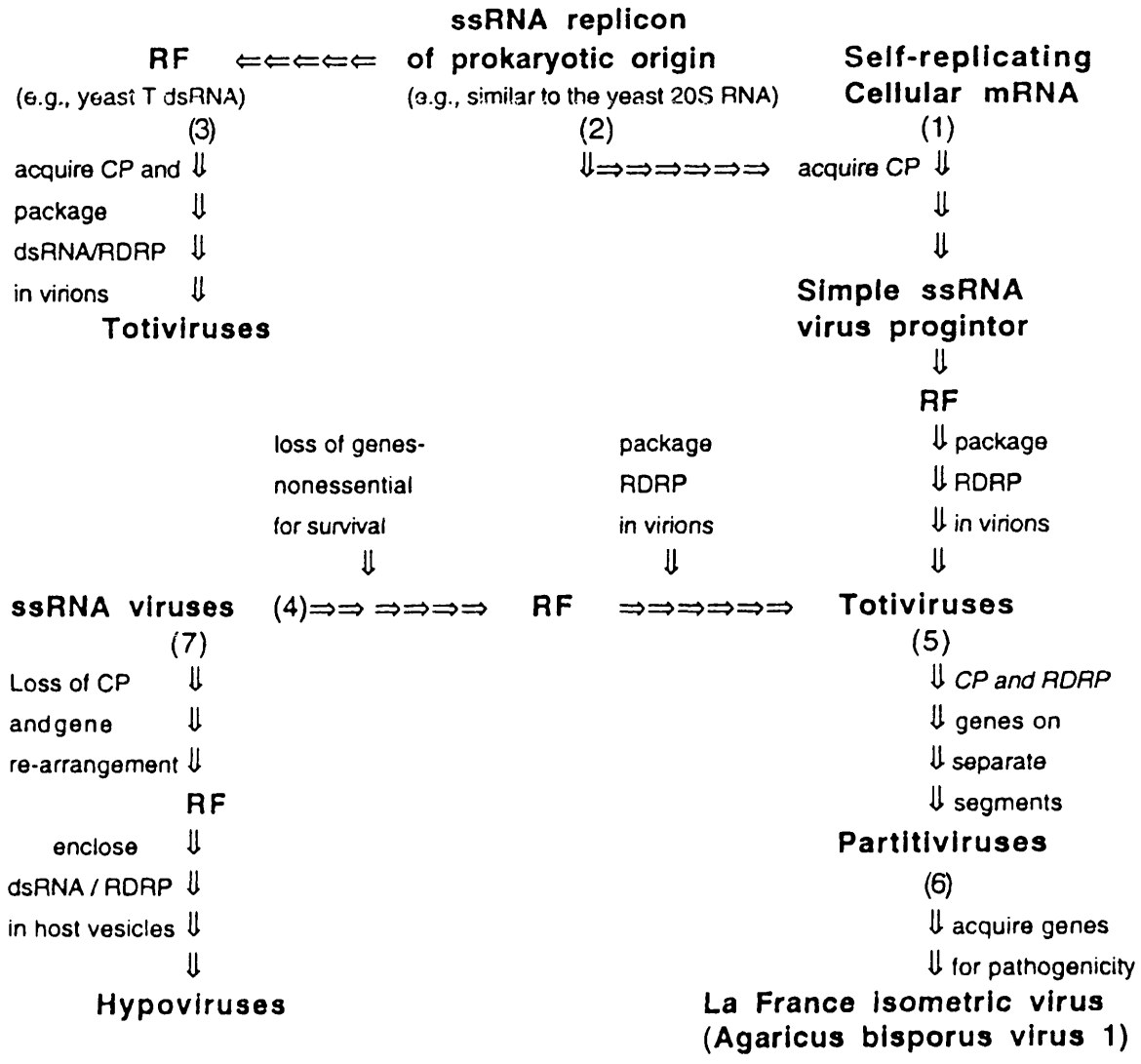


Fig. 1.2 Diagram showing possible pathways in the evolution of mycoviruses (Ghabrial, 1998).

The yeast 20S and 23S RNA replicons (Wickner, 1996) and their replicative form (Rfs) T and W dsRNAs may represent fossils of the ancestral RNA replicons that gave rise to the single and double stranded RNA viruses with simple genome(Fig. 1.2 pathways 2 and 3). Based on tentative phylogeny of RdRp of (+) strand RNA viruses, Koonin and Dolja (1993) grouped the 20S/23S RNAs (or their RFs, T/W dsRNAs with those of phage lineage under supergroup 2 of (+) strand RNA viruses. The regressive theory of virus origin (strauss *et al.*, 1996) may account for the occurrence of the 20S RNA replicons and similar RNA replicons of prokaryotic (phage) lineage in fungi. Because the totivirus RdRps were grouped with those of supergroup 1(+) strand RNA viruses (Koonin and Dolja, 1993) , one may envisage that the totiviruses have arisen from 20S-like RNA replicons with supergroup 1 lineage.

Totiviruses could also have evolved from progenitors with more complex genomes via reduction/ loss of genes not essential for survival (Fig. 1.2, pathway 4). Those who favour this pathway present as evidence known examples of closely related dsRNA viruses that differ in the number of dsRNA segments. Although very few segmented dsRNA viruses have been characterized at the molecular level, it appears that the lost segments in question represent defective or satellite dsRNAs. Thus, no viral sequences (gene) were lost since the defective dsRNAs represent redundant sequences and satellite dsRNAs contain sequences unrelated to the virus.

The partitiviruses may have evolved from totiviruses by dividing their genomes between two dsRNA segments (Fig. 1.2, pathway 5). Like the totiviruses, the partitiviruses have simple genomes comprised of two genes (CP and RdRp) but, unlike the totiviruses, each gene is carried on a separate dsRNA segment (bipartite genomes). Whereas many of the totiviruses express their RdRp as a CP-RdRp fusion protein by fusing the CP and RdRp ORFs, the partitiviruses express their CP and RdRp as separate proteins from their divided genomes. The totiviruses (e.g. Hv 190SV) that express their RdRp as a separate nonfused protein, via an internal initiation mechanism (Huang and Ghabrial, 1996), may be intermediates in the evolution of partitiviruses from ancestral totiviruses.

La France Isometric Virus : LIV (*Agaricus bisporus*)

The majority of virions isolated from La France diseased fruit bodies and mycelium are isometric 25 , 34-36 nm in diameter and co-purify with 9 dsRNAs (referred to as disease-associated dsRNAs). The size of dsRNA segments varies from 0.78 kb to 3.6 kb, three of which are believed to be satellites (Ghabrial, 1994 and Harmsen, *et al*, 1991). Although present evidence strongly suggests that the 9 disease-specific dsRNA are encapsidated in 34-36 nm isometric virus particles (Goodin, *et al.*, 1992), it is not clear whether the dsRNAs are encapsidated individually, in various combinations, or all nine segment are packaged in single particles. Judging from the size of the particle (34- 36 nm), it is highly unlikely that all dsRNAs are packaged together in single particles.

More likely, LIV represents a multiparticle system in which the various particle classes have similar densities. Packaging of the dsRNA segments probably occurs by a headful-type mechanism, in which either various combinations of different dsRNA segments, or single/multiple copies of the individual dsRNAs, dependent on size of segment, are packaged together. The various particle classes are thus predicted to have similar densities, and this is consistent with the results of caesium sulphate density gradient analysis of the purified virions (Goodin, *et al.*,1992).

The amino acid sequences have now been deduced from the nucleotide sequences of five of these dsRNAs, and only one of the predicted proteins (RdRp) shows homology to proteins in current databases (Van der Lende, *et al.*, 1996 and Harmsen, *et al.*, 1991). The LIV RdRp is most closely related to those of totiviruses and partitiviruses. However, unlike the totiviruses and partitiviruses, LIV is believed to be of recent origin since its codon usage differs from that of its host *A. bisporus* (Van der Lende, *et al.*, 1996). Because of its genome nature (segmented dsRNA genome) and RdRp sequence similarity, it may be possible that LIV arose from a partitivirus by acquiring additional gene (via reassortment/ recombination) that are essential for its spread in the mushroom fleshy tissue and for pathogenicity (Fig 1.2, pathway 6) ; (Ghabrial, 1998).

1.3 DSRNA VIRUSES IN *AGARICUS BISPORUS*

The edible fungus *A. bisporus* is commercially cultivated on a large scale for mushroom production. The differentiation process leading to these reproductive structures is influenced by available nutrients and abiotic factors such as temperature, humidity and the carbon dioxide concentration. By manipulating environmental conditions during cultivation, the onset and further development of fruit body formation can be controlled to optimize mushroom yields (Fig 1.3). The mushroom industry is sensitive to crop losses caused by bacterial, fungal infections and losses caused by the formation of ill-shaped or malformed mushrooms (Sonnenberg, *et al.*, 1995 a).

In 1948 a serious disease of the cultivated mushroom *A. bisporus* , characterised by malformed fruiting bodies and loss of crop, was first reported in a mushroom house owned by the La France brothers of Pennsylvania (Sinden and Hauser, 1950). The disease was termed La France disease, and similar afflictions were reported soon afterward from England, France, The Netherlands, Italy, Denmark, Japan, and Australia. Different designations, such as "x-disease", "watery stipe", "brown disease", and "die-back", were given to disease essentially the same as La France. The significance of the 1948 outbreak lies in that it led to the discovery of fungal viruses. Hollings (1962) observed at least three types of virus-like particles in association with the fungus. Because the nature of genomes of the viruses involved was not elucidated, and because some apparently healthy mushrooms also harboured viruses ,earlier test for pathogenicity (Koch's postulates) were difficult to interpret. Furthermore, precautions against contamination with exogenous spores were not taken. Today, 40 years later, the evidence for viral aetiology is stronger, but remains equivocal (Ghabrial, 1994). La France disease or die-back disease of *A. bisporus* was associated with dsRNA viruses. The most common symptoms of La France are a delay in the appearance of sporophores , bare areas on the growing bed surrounded by mushrooms with long bent stipes and small caps and a severe loss in yield and quality of mushroom (Fig 1.4).

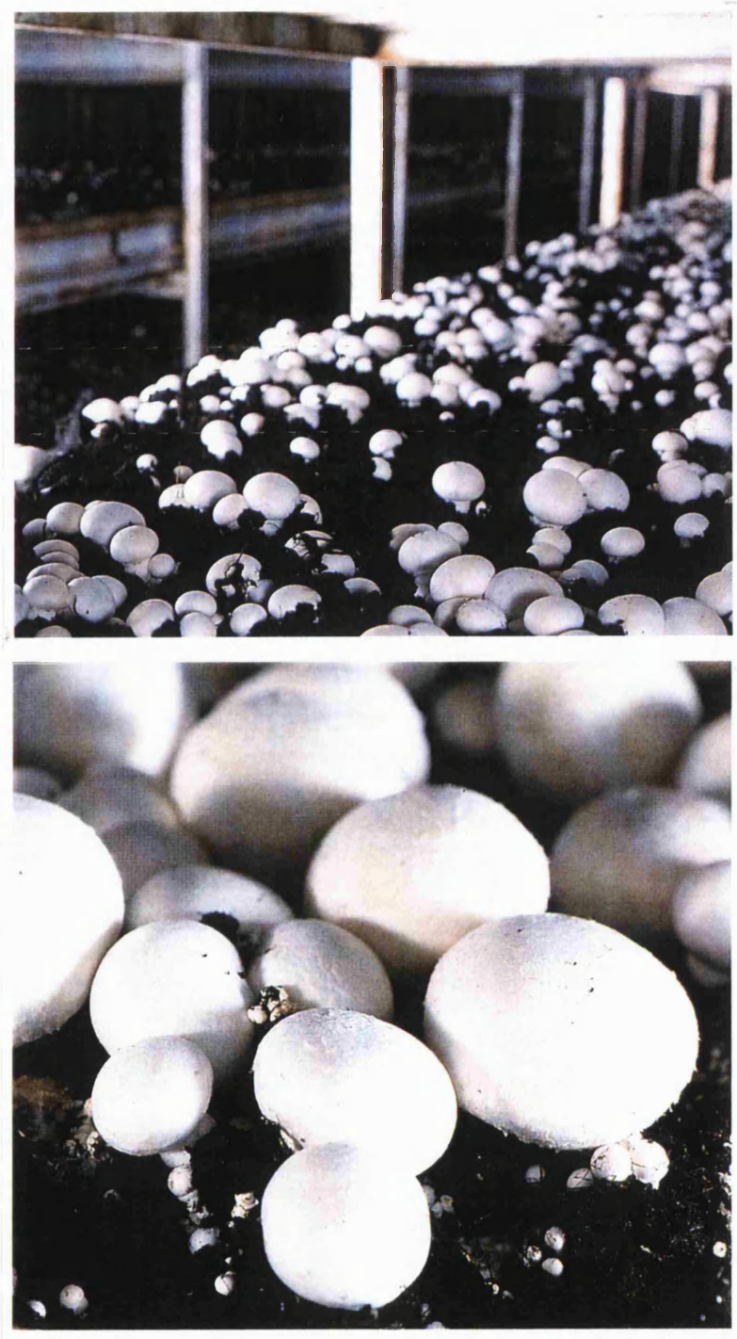


Fig 1.3 From top: mushroom *A. bisporus* beds in growing room ;
: mushroom *A. bisporus* ready for the first picking.
(Leckford estate mushroom)

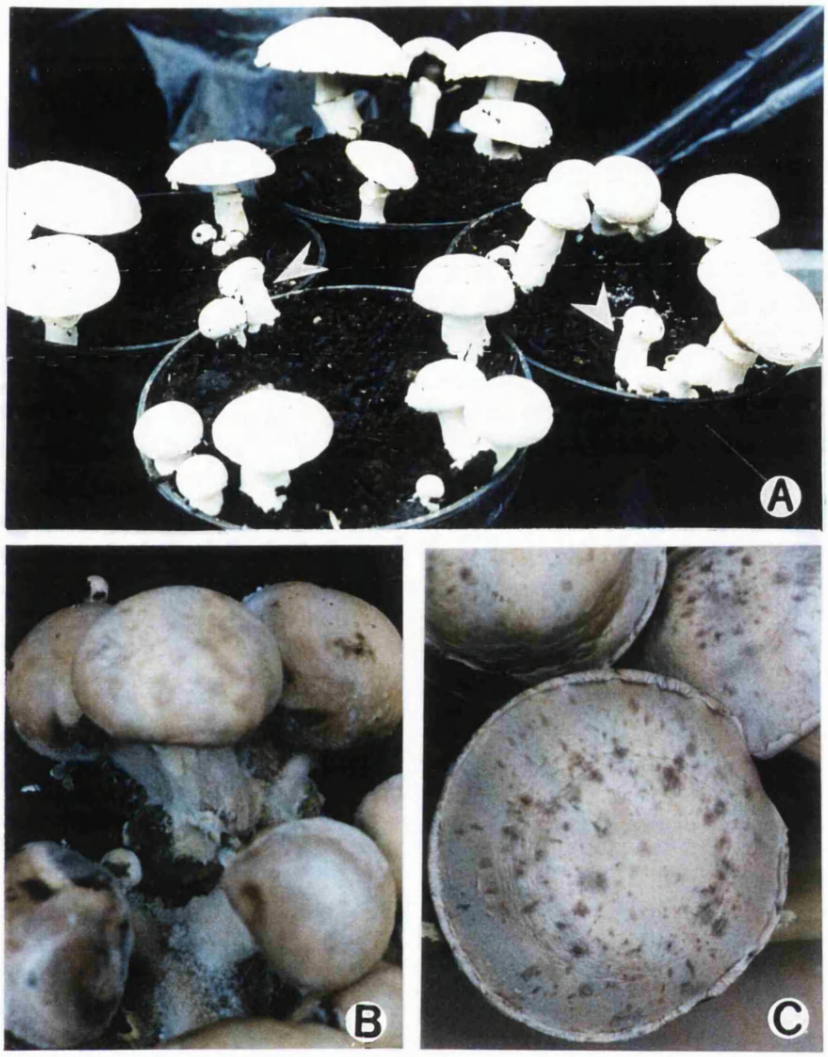


Fig. 1.4 A) : Mushroom *A. bisporus* with symptoms of virus disease ; long, slightly bent stipes and a veil which is too low . Small caps which open prematurely. Arrow show drumsticks that cap and stipe are nearly one.

B) : Mushroom with fungal infected caps and stipe showing light brown blotches, slightly sunken spots and compost colonized by green mould such as *Trichoderma* spp.

C) : Cap spotting caused by *Verticillium fungicola*. The spots are dark brown with a diffuse edge.

Three types of virus particles are consistently associated with mushroom virus disease; isometric particle of various diameters, 25 and 34 nm particles being the predominant types containing dsRNA (Van Zaayen, 1979 ; Frost and Passmore, 1980). In addition, a bacilliform particle containing ssRNA was found (Tavantzis, *et al.*, 1980; 1983 ; Revill, *et al.*,1994).

Three types of particles have been demonstrated in ultrathin sections of diseased fruit bodies. The number of 34 nm particles observed in sections was often much larger than was expected from purification experiments, hence these particles may be most important in mushroom virus disease (Van Zaayen, 1972). Moreover, the bacilliform particle has not been detected alone in infected mushrooms, and is always found in association with the isometric particle (Ghabrial, 1994).

Mushroom bacilliform virus (MBV) is the member of the family *Barnaviridae* (Romaine, 1995). The virion is a 19x50 nm bacilliform particle encapsidating a ssRNA positive-sense monopartite genome of 4 kb (Tavantzis, *et al.*, 1980 ;1983). MBV is not detected in healthy mushrooms and yet is not detected in all cases of La France disease (Van Zaayen, 1979; Romaine and Schlagnhaufer, 1995). The MBV genome has been sequenced and contains four major open reading frames (ORFs), encoding polypeptides of Mr 20,000, 73,000, 47,000, 22,000, respectively. (Revill, *et al.*, 1994).The arrangement of ORFs at the 5'end of the genome and the deduced amino-acid sequences of two of the putative gene products (putative serine protease and RdRp) show remarkable similarity to some plant viruses , particularly subgroup II luteoviruses ; pea enation mosaic virus :PEMV and the cowpea strain of southern bean mosaic virus : SBMV(Revill, *et al.*, 1995). Their results confirm that the similarity to plant viruses extends to the initiation of protein synthesis *in vitro* at the 5'end of the MBV genome where translation of a second ORF is initiated downstream of the start point for the first ORF and to the location of the coding sequence for the genome-linked protein (Vpg),which is the same as for the subgroupII luteovirus ,potato leaf roll virus : PLRV.

Romaine et al,(1994) showed three dsRNA were purified from fruit bodies of the *A. bisporus* : two major dsRNA segments of >13.1 kb (L-RNA) and 2.4 kb (S-RNA) and a minor segment of 5.2 kb (M-RNA). L-,M-,and S-RNAs co-purified with spherical fungal vesicles measuring approximately 75 nm in diameter. The three dsRNA were intimately associated with the vesicles as suggested by their lower buoyant density in caesium sulphate (1.27g/cc) compared to that of phenol-extracted dsRNA (1.42g/cc) and by their resistance to hydrolysis by ribonuclease at low ionic strength. Using a variety of conditions during purification, no virus-like particles were found to be associated with the dsRNAs. In Northern analysis, L-,M- and S-RNAs failed to cross-hybridize with the genomic dsRNAs of La France isometric virus. The largest and the smallest of the dsRNAs, L-RNA (>13.1 kb) and S-RNA (2.4 Kb), are identical in size to segments previously detected in total dsRNA fractions of healthy mushroom and mycelium (Harmsen, *et al.*, 1989 ; Romaine and Schlagnhaufer, 1989).

The dsRNA pattern associated with crop loss always consisted of 10 major dsRNA molecules. This pattern comprised six large dsRNAs (L1 = 3.6 kb , L2 = 3.0 kb, L3 = 2.8 kb , L4 = 2.7 kb , L5 = 2.5 kb , L6 = 2.35 kb), two medium - sized dsRNAs (M1 = 1.6 kb , M2 = 1.35 kb) and two small dsRNAs (S1 = 0.85 kb , S2 = 0.78 kb). Up to eight "minor" dsRNA molecules could often be seen on overloaded agarose gels (XL1 to XL4, approximately 15 kb; L0 = 6.5 kb, L7 = 2.0 kb, L8 = 1.9 kb, S3 = 0.27 kb). The ten major dsRNAs, ranging in size from 0.8 to 3.6 kb are individually unique since they do not cross-hybridise under stringent condition (Harmsen, *et al.*, 1989). For American diseased mushrooms, Mario, *et al* (1976) described the presence of only six dsRNAs, possibly corresponding to the L1 to L5 plus M2 segments described here. Wach, *et al.*, (1987) investigated American diseased fruit bodies in more detail and found a similar pattern.

Full - length transcripts of dsRNA L3 , L6 , M1 , M2 , S2 and S3 were detected in total ssRNA preparations from diseased mushrooms by hybridisation with cloned probes (Harmsen and Wessels , 1994). These results showed that at least six dsRNAs were actively transcribed in diseased sporophores. Whether these transcripts serve as mRNA or act as templates for dsRNA synthesis, or both, is not known.

Although the nine dsRNAs have unique nucleotide sequences (Harmsen, *et al.*, 1989) three of the segments (M1, S1 and S2) occur in submolar concentrations and could be satellite dsRNA. The bacilliform particles of the mushroom bacilliform virus (MBV) are not likely to package any of the ten dsRNA. Highly purified virions of MBV do not contain dsRNA, but rather a 4.4-kb ssRNA that shares no sequence homology with the disease-specific dsRNAs (Romaine and Schlagnhaufer, 1991). Moreover, The 25-nm isometric particles has been shown to have a single coat protein (CP) of 24.5 kDa (Barton and Hollings, 1979).

With respect to the translation of other individually isolated dsRNA, L1, L2, L4-L6, and M1 only L1 (3.6 kb) had sufficient coding capacity to encode a polypeptide of 130-kDa, as resolved in translation of total dsRNA. Based on its size, the 130-kDa polypeptide is a candidate to comprise the viral RdRp. It is of interest in this regard that a minor protein of 129 kDa has been reported to copurify with the 36-nm virions form diseased mushroom (Goodin, *et al.*, 1992).

Van der Lende, *et al.*, (1994) assumed that the viral proteins of molecular weight 120 K, 115 K and 90 K were encoded by the disease-specific dsRNA. The L1 dsRNA (3.6 kb) and possibly the L2 dsRNA (3.0 kb) may contain sufficient coding capacity to encode the 120 K and 115 K protein, particularly since the apparent high molecular weight of these proteins on the gel may be due to post-translational modifications, as was shown for mycovirus protein found in *Helminthosporium victoriae* (Ghabrial, *et al.*, 1987; Ghabrial and Havens, 1992). The protein of molecular weight 90 K could be encoded by any one of the L dsRNAs but M1, M2, S1 and S2 appear to lack sufficient coding capacity.

Van der Lende, *et al.*, (1996) reported the sequencing of clones corresponding to L1 and L5 dsRNA. The deduced amino acid sequence of L1 dsRNA (1078 amino acid, molecular weight 121 k) showed significant homology with RdRp of other dsRNA viruses. The deduced amino acid sequence of L5 dsRNA (724 amino acid , MW 82 kDa) showed no homology with known proteins. Amino acid sequences of tryptic digests of three virion-associated proteins were determined.

The 34 nm virion-associated protein of molecular weight 115 k was encoded by the L1 dsRNA, thus identifying this protein as the RdRp. Only the L1 dsRNA showed homology to proteins in current protein databases. It encodes an RdRp based on the presence of the consensus sequences found in other RdRp. Best matching sequence occur in dsRNAs of virus of the ascomycete *Saccharomyces cerevisiae* , the heterobasidiomycete *Ustilago maydis*, and protozoans *Leishmania braziliensis* subsp *guyanensis* and *Giardia lamblia*. Contrary to the 36 nm isometric particles in *A. bisporus*, these viruses have all their genetic information present on one segment of dsRNA , encoding an RdRp and a capsid protein , and are classified as Totiviridae. Other fungal viruses contain two and sometime three dsRNA segments encoding at least a polymerase and a capsid protein and are called Partitiviridae. (Buck,1986). The 36 nm isometric particles in *A. bisporus* can not be considered a member of either the *Totiviridae* or the *Partitiviridae* as its genetic information is present on more than three separate dsRNA molecules (Van der Lende, *et al.*, 1996).

1.4 FUNGI ASSOCIATED WITH *AGARICUS BISPORUS*

Various fungi are known to be parasites of the cultivated mushroom. They are frequently recognised by their spore-producing structures, or by the symptoms shown by the affected crop. Most of the damaging fungal pathogens attack the sporophores and not the mushroom mycelium. Generally, the earlier the attack, the more distorted will be the ultimate mushroom. The incidence and severity of fungal pathogens varies from time to time, for example *Verticillium fungicola* var. *fungicola* , *Mycogone perniciosa, Cladobotryum dendroides, Dichliomyces microspores* , *Trichoderma harzianum*, have become more common (Fletcher, *et al.*, 1989). The study of mycoflora related to mushroom was shown that a great number of fungi , such as *Penicillium*, *Alternaria* and *Trichoderma* and some Mucorales, were found in mushroom spawn (Gea, *et al.*, 1995).

The relationships between *A. bisporus* and some of the fungi that can occur in mushroom crops are only partly understood. Many of these fungi become established because the physical and chemical environment of a poorly prepared compost is favourable for their development. Some fungi grow together with the mushroom mycelium and compete for nutrients.

Verticillium is probably the most common and serious fungal disease of the commercial mushroom crop and causes a disease commonly known as *Verticillium* brown spot or dry bubble . If left uncontrolled, it can totally destroy a crop in 2-3 weeks. The disease is caused by the fungus *V. fungicola* (syn. *V. malthousei*), although a closely related fungus, *V. psalliotae*, causes some similar symptoms. Recent work has shown that there are other varieties of *V. fungicola*. A second form, *V. fungicola* var. *aleophilum*, may be more commonly associated with crops of *Agaricus bitorquis* or may occur on *A. bisporus* when ambient temperatures are high. Infection of *A. bisporus* by *V. fungicola* results in brown lesions, shattered stipes and undeveloped (mummified) sporocarps. The degree of disease and the particular symptoms observed are dependent on the inoculum level and the point of development of the mushroom crop at the time of infection (North and Wuest, 1993).

Trichoderma is characterised by the production of very large quantities of dark green spores and it is these that are seen during various stages of cropping. A number of different species and strains of *Trichoderma* are found in mushroom culture , some harmless and others very damaging (Doyle, 1991). The relationship between the various *Trichoderma spp.* and the mushroom is not fully understood and almost certainly varies with the species and strain, but some are known to be pathogenic (Komatsu, 1976). The genus is well known for its mycoparasitic ability and for the production of toxins and antibiotic. The species most frequently associated with the mushroom crop are *Trichoderma aureoviride, T. harzianum, T. koningii, T. pseudokoningii, T. viride, T. longibrachiatum* (Seaby, 1987).

One of three biological forms of *T. harzianum* Rifai (group 2 of Muthumeenakshi, *et al.*, 1994) has been recognized as the main agent responsible for green mould epidemics in mushroom industries of the British Isles. Of two other *T. harzianum* forms, Th1 also has been located in mushroom compost but is generally not as aggressive a colonizer as Th2. *T. harzianum* Th3 is found in compost and the environs of mushroom production units but does not actively compete with *A. bisporus* (Seaby, 1987).

Molecular characterization of these three forms of *T. harzianum* using RFLP and PCR-based analyses revealed considerable genetic variation (Muthumeenakshi, *et al.*, 1994). *T. harzianum* group 2, which hitherto has not been identified from any source other than mushroom compost is genetically uniform. This homogeneity in *T. harzianum* group 2 supports the hypothesis that the green mould agent throughout the British Isles may have originated from a single source, possibly in Northern Ireland (Morris, *et al.*, 1995), although minor variation in mtDNA can distinguish Irish strains from those in Great Britain (Muthumeenakshi, *et al.*, 1994).

Trichoderma spp, common soil fungi are the most extensively studied and applied fungal biocontrol agents. The antifungal mechanisms by which these fungi function as biopesticides are not completely understood, and substantial differences in the mode of action and biocontrol activity have been found among different *Trichoderma* spp. during their interaction with various plant pathogens (Haren, *et al.*, 1996). Cell wall-degrading enzymes, antibiotics, and other factors induced in the presence of the host fungus are thought to be directly involved (Lorito, *al et* ., 1996 and Lorito, *et al.*, 1998). On the other hand, *Trichoderma harzianum* remains a potential threat to the mushroom industry following the green mould epidemic in the British Isles during 1985-86 and more recently in late 1990 and 1991. Losses have been estimated at $ 3-4 million to the UK and Irish mushroom industries (Fletcher, 1990).

1.4.1 dsRNA Viruses of Associated Fungi

It has become apparent in recent years that viral infections are not uncommon in fungi. Well-documented examples in which viruses or dsRNA have been associated with altered fungal phenotypes include the killer systems in *Saccharomyces cerevisiae* and *Ustilago maydis* and the "La France" disease of *A. bisporus* (Van Zaayen, 1979). Viruses or virus like particles (VLPs) have been reported in over 30 species of plant pathogenic fungi (Koltin and Levine, 1979). In several cases , the effects of the virus on pathogenicity of the fungus have been studied. Viruses seem to play little or no part in determining the pathogenicity of *Gaeumannomyces graminis, Periconia circinata, Colletotrichum lindemuthianum, Fusarium culmorum, Puccinia graminis,* and *sclerotium cepivorum* (Hollings, 1978).

Other economically important pathogens that contain dsRNA include *Sclerotinia sclerotiorum* (Boland, 1992), *Pythium irregulare* (Gillings, *et al.*, 1993), *Phytophthora infestans* (Newhouse, *et al.*, 1992 and Tooley, *et al.*, 1989) *Rhizoctonia solani* (Bharathan and Tavantzis, 1990) and *Verticillium albo-atrum* (Barbara, *et al.*, 1987). In some of these fungi , the presence of dsRNA apparently affects growth and virulence (Boland, 1992 ; Tooley, *et al.*, 1989).

Vagvolgyi, *et al.*, (1998) reported that the presence of dsRNA elements was examined in 123 strains representing 18 *Mucor* spp. These genetic elements were found to be present in 6 strains: 1 *M. aligarensis*, 1 *M. hiemalis*, 2 *M. corticolus*, 1 *M. mucedo* and 1 *M. ramannianus*. Electrophoretic separation of the nucleic acids revealed 4 different RNA patterns, with 1 to 5 discrete dsRNA bands. The molecular weights corresponding to these bands were 1.42 -4.15 kb.

Verticillium spp. remain one of the major threats to *A. bisporus* production throughout the world. Little is known about the pathogen diversity or the interaction between pathogen and host. Symptoms are varied and are thought to depend on a number of factors such as developmental stage, the time of infection and genetic

variability of the host and pathogen. Mycovirus infection has been reported in *Verticillium fungicola* with three types of particle measuring, about 48 nm , 35 nm and 35x17 nm in diameter (La Pierre, *et al.*, 1973). Recent work by Barbara, *et al.*, (1987) reported two isolates of *V. albo-atrum* infected ,each with two segments of dsRNA. The two dsRNA segments were electrophoretically indistinguishable and had molecular weights of approximately 1.16×10^6 and 1.32×10^6 daltons, respectively.

Viruses have been found in fungi parasitic on mushroom including *Mycogone perniciosa* (Lapierre, *et al.*, 1972), *Gonatobotrys* sp. (Spire, *et al.*, 1972), but here again there has been no evidence for the possible role of the these fungi as mycovirus vectors. However, mycovirus transmission through vectors remains a possibility. Potential vectors such as insects, mites, nematodes and hyperparasitic fungi have been investigated but the transmission of mycoviruses through vectors has not yet been proven (Lecoq, *et al.*, 1979).

1.5 AIMS OF THE PROJECT

The aims of this research are to characterise at the molecular level dsRNAs isolated from ; *A. bisporus* , *V. fungicola* and *T. harzianum* and to investigate a possible relationship between the dsRNAs from these fungi.

CHAPTER 2

Isolation of dsRNA

CHAPTER 2 Isolation of dsRNA

2.1 INTRODUCTION

In the last three decades, fungal double-stranded RNA (dsRNA) elements have been the subject of considerable research because of their potential adverse effects on plant pathogenic fungi, and the prospects of utilizing them in biocontrol schemes against the host fungus. DsRNA elements are found either packaged in protein capsids (mycovirus), or as unencapsidated dsRNA molecules. The dsRNA mycoviruses may have segmented or unsegmented genomes , whereas the unencapsidated dsRNA elements usually consist of multiple segments and are often associated with cell membranes (Nuss and Koltin 1990, Ghabrial, 1994). Most of these have genomes of dsRNA ,but genomes of single-stranded RNA (ssRNA) and DNA have also been described (Buck, 1986).

Among the edible fungi *Agaricus bisporus* (J.E. Lange) Imbach holds a unique position. Fruit bodies of this basidiomycete are cultivated in large amounts and used as a vegetable crop. Cultivation started in France over 200 years ago and in the last few decades mushroom production has grown into a large industry which has expanded all over the world. Its production exceeds 1.8×10^6 tons per year, $8\text{-}9 \times 10^5$ of these are cultivated in Europe (ca. 2 billion Euros) .The Netherlands and France are the countries with the highest production of mushrooms (2.4 and 1.6×10^5 tons, respectively), followed by the UK, with an annual production of ca. 10^5 tons (Soler-Rivas, *et al.*, 1999)

Due to the relative difficulty of breeding of *A. bisporus*, most of the strains that are used nowadays for commercial cultivation are identical to or derived from only two "hybrid" strains, Horst U1 and Horst U3, which were developed by Fritsche (1983). The lack of genetic variation between the commercial mushroom strains forms a severe threat to the mushroom industry (De Groot, *et al.*, 1998).

The industry is sensitive to crop losses caused by bacterial and fungal infections and losses caused by the formation of ill-shaped or malformed mushrooms (Sonnenberg, *et al.*, 1995a). La France disease or Die-Back disease of *A. bisporus* was first described after a severe outbreak at a commercial farm located in southeastern Pennsylvania (Sinden and Hauser,1950). Today the disease occurs in most mushroom-growing countries (Van Zaayen, 1979). The most common symptoms are a delay in the appearance of sporophores , bare areas on the growing bed surrounded by mushrooms with long bent stipes and small caps and a severe loss in yield and quality of mushroom. Symptoms are associated with the presence of virus particles (25 and 34 nm ; Van Zaayen, 1979) and 9 major dsRNAs (Harmsen, *et al.*, 1989).

The genus *Verticillium* Nees contains a heterogeneous group of asexual fungi, many of which are of considerable importance in agriculture as pathogens of plants, insects, and nematodes. The two most commercially important of the *Verticillium* plant pathogens, *V. albo-atrum* and *V.dahliae*, cause vascular wilts in many hosts worldwide (Rowe, 1995). Several other *Verticillium* species are being studied as potential biocontrol agents for insect and nematode pests. These *Verticillium* spp. have a wide host range with little host specificity. Thus, the insect pathogen *V. lecanii* and the nematode pathogen *V. chlamydosporium* are hyperparasites on other fungi (rust, powdery mildew fungi).

The pathogens of fungi, *V. fungicola* and *V. lamellicola*, are also pathogenic to mites and insects (Bidochka, *et al.*,1999). The insect pathogens and mushroom pathogen (*V. fungicola*) were characterized by the production of high levels of subtilisin-like proteases active against a chymotrypsin substrate (succinyl-Ala2- Pro-Phe-NA) and the inability to clear pectin. The insect and mushroom pathogens, and several nematode pathogens, were distinguishable from the plant pathogens by their ability to produce chitinases. Broad-spectrum subtilisins are the major proteins produced by the insect pathogens *Metarhizium anisopliae* and *Beauveria bassiana* during infection processes, and have much greater ability than the trypsin-like enzymes to degrade insect cuticle (Bidochka, *et al.*,1999).

V. fungicola is an important fungal pathogen of the mushroom, *A. bisporus* and the causal agent of the disease commonly known as *Verticillium* brown spot or dry bubble. Infection of *A. bisporus* by this pathogen results in brown lesions, shattered stipes and undeveloped (mummified) sporocarps. The degree of disease and the particular symptoms observed are dependent on the inoculum level and the point of development of the mushroom crop at the time of infection (North and Wuest, 1993).

Trichoderma green mould has been associated with *A. bisporus* culture since the mushroom was first domesticated in France. Green mould has caused periodic losses to growers over the years, but they have learned to manage this malady through ecological , cultural, and chemical means. Within the last decade, however, growers in Ireland, England, Canada and the United States have experienced outbreaks of *Trichoderma* green mould that have resulted in millions of dollars in crop losses. In Pennsylvania alone, crop losses are estimated at more than $20 million (Ospina-Giraldo, *et al.*, 1998).

On the other hand, *T. harzianum* has been shown to act as a mycoparasite against a range of economically important aerial and soil-borne plant pathogens. Different factors involved in the antagonistic properties of *Trichoderma* have been identified, including antibiotics and hydrolytic enzymes such as β-(1,3) glucanases, proteases and chitinases (Geremia, *et al.*, 1993) The initial interaction between *Trichoderma* and its host is characterized by the chemotropic growth of hyphae of the mycoparasite towards the host. When the mycoparasite reaches the host, its hyphae often coil around it or are attached by hook-like structures (Elad, *et al.*, 1983b). Following these interactions, the mycoparasite penetrates the host mycelium, apparently by partially degrading its cell wall. Susceptible host mycelia show rapid vacuolation, collapse and disintegration (Elad, *et al.*, 1983a ; Benhamou and Chet 1993).

The aims of this chapter were to determine the incidence of dsRNA in isolates of *A. bisporus* , *Verticillium* spp. and *T. harzianum* from different geographic origins.

2.2 MATERIALS AND METHODS

2.2.1 Source and maintenance of culture

Mushroom *A. bisporus* (isolates V95 and EU) with symptoms of La France disease were obtained from infected commercial crop (MAFF, York UK ; and farm in UK, respectively). Cultures of these infected mushrooms and an apparently healthy control were prepared and maintained on slant tubes of 1% malt extract agar (MA). Six isolates of *Verticillium* spp originally isolated from *Agaricus* sp. were collected from CBS, The Netherlands. Fifteen isolates of *T. harzianum* isolated from *Agaricus* spp. were obtained from HRI ,Wellesbourne UK. These were maintained on slant tubes of 1% Malt agar.

2.2.2 Stationary liquid culture

Mycelium of *Agaricus* was grown in stationary liquid culture as follows : 100 ml of medium broth (2% malt extract, 0.5% mycological casein, 0.5% olive oil and 0.2% KH_2PO_4) in a 250 ml flask was inoculated with four 6 mm-diameter mycelial plugs and maintained at 25°C without agitation. Cultures were incubated for 10 weeks, and harvested by vacuum - filtration and stored at -20 °C.

Mycelium of *Verticillium* and *Trichoderma* were cultured in stationary liquid culture as follows: 20 ml of medium broth (2% malt extract, 2% mycological peptone) contained in a Petri dish were inoculated with two 6 mm-diameter mycelium plugs and maintained at 25 °C without agitation. Cultures were incubated for 1 week, harvested and stored at -20 °C.

2.2.3 Extraction and purification of dsRNA

A modification of the method outlined by Jordan and Dodds (1985) was used to isolate dsRNA . Four grams of fruit body or fungal mycelium were finely ground with a pestle in a mortar containing liquid nitrogen. The resulting powder was transferred to a 35 ml centrifuge tube, and the following were added : 4 ml extraction buffer [2 x STE buffer (Valverde, *et al.*, 1990), 2% SDS (sodium dodecyl sulphate) , 1% PVP-10 (polyvinyl pyrrolidone-10)] , 4 ml water-saturated phenol and 2 ml of chloroform : pentanol (24 : 1 , v/v). The mixture was shaken gently for 20-30 min at room temperature. The tube then was centrifuged at 4,000 g for 20 min and the aqueous phase was recovered and adjusted to 16 % ethanol.

The adjusted aqueous phase was added to 1 g of CF-11 cellulose (Whatman). The tube was vortexed 3 times in 30 min and then centrifuged at 4,000 g for 10 min to pack the cellulose - bound dsRNA. The CF-11 cellulose was washed 2 times by adding 5 ml 16% ethanol in STE , vortexing, centrifuged at 4,000 g for 10 min and decanting the supernatant. After the final wash , the dsRNA was eluted by adding 5 ml 1x STE to the cellulose pellet , vortexing , centrifuging at 4,000 g for 20 min and transferring the dsRNA-containing supernatant to a new tube.

The dsRNA was concentrated by ethanol precipitation by adjusting the samples to 67% ethanol (v/v) and 50 mM sodium acetate, and storing them for at least 4 hr at -20 °C , and pelleted by centrifugation at 4,000 g for 60 min. The dsRNA pellet was resuspended in 400 µl of distilled water and transferred to a 1.5 ml Eppendorf tube for further processing.

2.2.4 Gel electrophoretic analysis dsRNA

Purified dsRNA was analysed on 0.8 % agarose gel in TBE buffer pH 8.3 (Sambrook, *et al.*, 1989) and EtBr (0.5 µg/ ml) was added. The dsRNA samples were resuspended in loading buffer : 0.1x loading buffer / sample (Valverde, *et al.*, 1990). Gels were run at 60 volts for 2.5 hours. Nucleic acid bands were visualised by transilluminater UV light and photographed (Life sciences ; UVP Ltd, UK). Molecular weight markers for agarose electrophoresis were a DNA 1 kb ladder (Promega).

2.2.5 Ribonuclease treatment

To confirm the dsRNA nature, the nucleic acid was treated with ribonuclease to be sure that only dsRNA was ultimately observed. The samples of this material were treated with various nucleases to identify their nature. The reaction conditions of RNase A and DNase I were as described by Sambrook, *et al.*,(1989).

The pancreatic RNase A (1 µg /ml ; Sigma) digestions were carried out in the following high- and low- ionic strength buffers; in 20 µl of 2xSSC (1xSSC = 0.15 M NaCl, 0.015 M Sodium citrate pH 7.0) and 0.1xSSC at 37°C for 10 min, respectively.

Treatment with 1 unit DNase I RNase free (Promega) was performed in 20 µl in 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$ for 10 min at 37°C. All samples were analysed by electrophoresis on 1% agarose gels.

2.3 RESULTS

2.3.1 Isolation and characterization of dsRNA

A. bisporus

Multisegment dsRNAs were detected in V95 and EU strains of sporophores and mycelia of *A. bisporus* by agarose gel electrophoresis of preparations purified by CF-11 cellulose chromatography and stained with ethidium bromide (Table 2.1, Fig 2.1). Electrophoresis of dsRNA from V95 in agarose gels separated the dsRNA into eight segments (Fig. 2.1; lane 1) with estimated molecular weights of 3.6, 3.0, 2.8, 2.7, 2.5, 1.5, 1.3, and 0.85 kb (L1-L5, M1-M2, S).The dsRNA of EU strains consisted of eleven segments (Fig. 2.1; lane 2) with apparent molecular weights of 3.6, 3.0, 2.8, 2.7, 2.5, 2.3, 1.5, 1.3, 1.0, 0.85 and 0.78 kb (L1-L6, M1-M3, S1-S2). dsRNA was not detected in an apparently healthy control strain of *A. bisporus* (Table 2.1).

It was confirmed that all bands were of dsRNA as the material was resistant to RNase A at high ionic strength (2xSSC) buffer and DNase I , but sensitive to RNase A at low ionic strength (0.1xSSC) buffer (Fig 2.2).

***Verticillium* spp.**

Among the six *Verticillium* spp. isolates tested only *V. fungicola* (V7-3 ,The Netherlands) and *V. psalliotae* (V5-2, New Zealand) contained detectable amounts of dsRNA (Table 2.2). Five dsRNA segments were found in *V. fungicola* strain V7-3 with apparent molecular weights about 2.4 -1.1 kb (Fig 2.3.; lane 1) .Their sizes were approximately 2.4 (dsRNA 1) , 2.1 (dsRNA 2), 2.0 (dsRNA 3) , 1.4 kb (dsRNA 4) and 1.1 (dsRNA 5) as determined by 0.8% agarose gel electrophoresis. Only one dsRNA segment was detected in *V. psalliotae* isolate V5-2 with apparent molecular weight about 0.7 kb (dsRNA 1) (Fig 2.3; lane 2). It was not detected in three strains of *V. fungicola* (V7-4, V115-1, V61-7) and *V. psalliotae* (V7-13) (Table 2.2).

Table 2.1 List of analyzed fungal dsRNA of *A. bisporus.*

Geographic origin		dsRNA	No.# band
United Kingdom	(V95)	+	8
Europe	(EU)	+	11
Apparently healthy control		-	-

Table 2.2 List of analyzed fungal dsRNA of *Verticillium* spp.

Species / isolate (isolated from *Agaricus* spp.)		Geographic origin	dsRNA (No. # band)
V. fungicola var. *fungicola*	V7-4	The Netherlands	-
V. fungicola var. *fungicola*	V115-1	England	-
V. fungicola var. *aleophilum*	V7-3	The Netherlands	+ (5)
V. fungicola var. *aleophilum*	V61-7	The United States	-
V. psalliotae	V7-13	Israel	-
V. psalliotae	V5-2	New Zealand	+ (1)

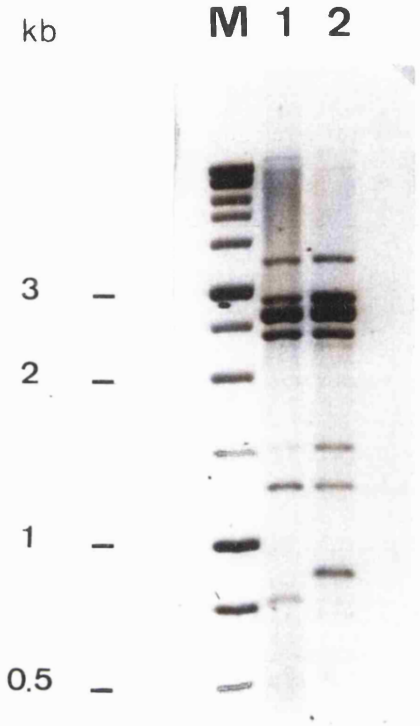


Fig. 2.1 0.8% Agarose gel electrophoresis of dsRNA extracted from mycelium isolates of *A. bisporus* by CF-11 chromatography. Lanes are as follows:

M = Molecular weight marker (kb DNA Ladder)

1 = *A. bisporus* isolate V95 from UK

2 = *A. bisporus* isolate EU

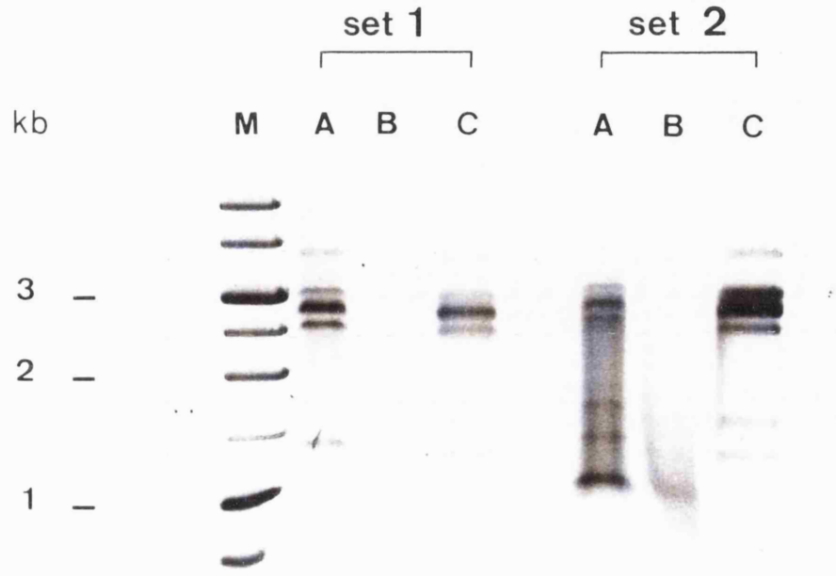


Fig. 2.2 Agarose gel electrophoresis of enzyme-digested components from two *A. bisporus* isolates :

Set 1 = *A. bisporus* isolate V95 from UK

Set 2 = *A. bisporus* isolate EU

Lane M : Molecular weight marker (kb DNA ladder)

Lane A : dsRNAs digested with 0.5 μg/ml RNase A in 2 x SSC for 10 min at 37 °C.

Lane B : dsRNAs digested with 0.5 μg/ml RNase A in 0.1 x SSC for 10 min at 37 °C

Lane C : dsRNAs digested with 1 unit DNase I in 40 mM Tris -HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$ for 10 min at 37°C.

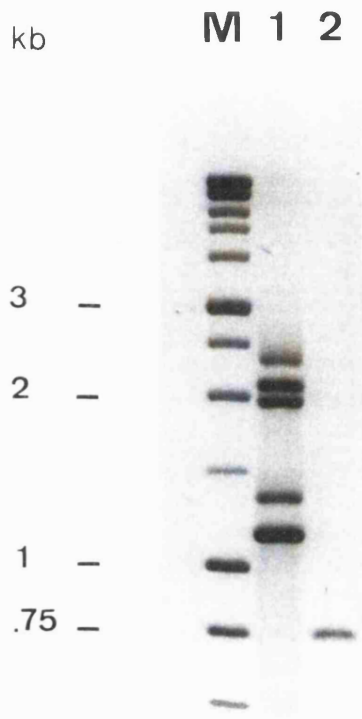


Fig. 2.3 0.8% Agarose gel electrophoresis of dsRNA extracted from mycelial isolates of *Verticillium* spp. by CF-11 chromatography. Lanes are as follows:

M = Molecular weight marker (kb DNA Ladder)

1 = *V. fungicola* isolate V7-3

2 = *V. psalliotae* isolate V5-2

V. fungicola isolate V7-3 and *V. psalliotae* isolate V5-2 exhibited individual differences in dsRNA patterns , numbers and sizes. None of these features changed after prolonged maintenance or repeated subculturing of these fungi. The dsRNA nature was confirmed by insensitivity to RNase A at high ionic strength (2xSSC) buffer and DNase I , but sensitive to RNase A at low ionic strength (0.1xSSC) buffer (Fig 2.4).

T. harzianum

Of the fifteen isolates of *T. harzianum* that were examined, six had dsRNA (Table 2.3). Four dsRNA fragments were evident from *T. harzianum* isolates T7, KPNT, T32, Th1c. Their sizes were approximately 2.0 (dsRNA 1) , 1.95 (dsRNA 2), 1.8 (dsRNA 3) and 1.65 kb (dsRNA 4) . Two dsRNA fragments were detected in Th3c and A006022. Their sizes were approximately 2.0 (dsRNA 1) , 1.95 (dsRNA 2) as determined by gel electrophoresis.

All of the isolates that contained dsRNA were assigned to two groups based on their general similarities in the banding pattern as group I (4 fragments) and group II (2 fragments) (Fig 2.5). It was not detected in nine strains of *T. harzianum* ; T28JF, T7R, Th1M, Th2A, Th2F, TD15, BE, Rmioc, Rmion (Table 2.3).

Such molecules were completely degraded by RNase A treatment in a low-ionic strength buffer (0.1xSSC), but they were resistant to DNase I. Furthermore, they were resistant to RNase A in a high-ionic strength buffer (2xSSC) (Fig 2.6). Thus, these results clearly show that these genetic elements are composed of dsRNA.

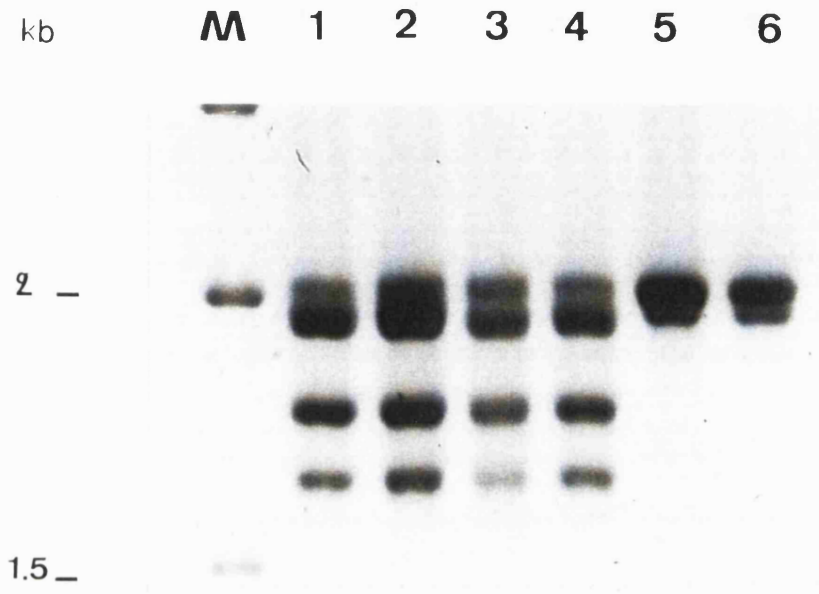


Fig. 2.5 0.8% Agarose gel electrophoresis of dsRNA extracted from mycelial isolates of *T. harzianum* by CF-11 chromatography. Lanes are as follows:

M = Molecular weight marker (kb DNA Ladder)

1 = T7; 2 = KPNT; 3 = T32

4 = TH1C; 5 = TH3C; 6 = A006022

Table 2.3 List of analyzed fungal dsRNA of *Trichoderma harzianum*.

Geographic origin	dsRNA	No.# band.
England :		
KPNT	+	4
T28 JF	-	-
T32	+	4
T7	+	4
T7 R	-	-
Northern Ireland:		
Th1 C	+	4
Th1 M	-	-
Th2 A	-	-
Th2 F	-	-
Th3 C	+	2
TD 15	-	-
Republic of Ireland:		
A006022	+	2
The United States / Canada:		
BE	-	-
Rmioc	-	-
Rmiom	-	-

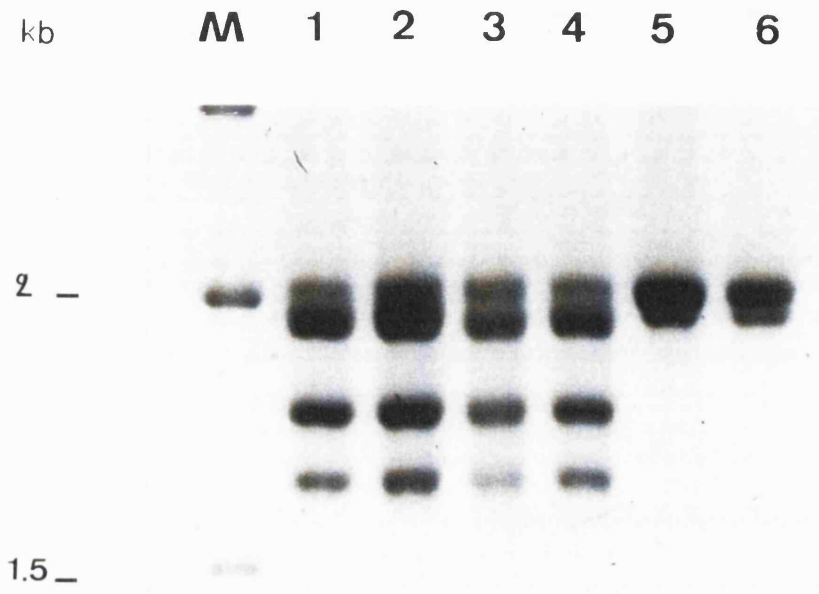


Fig. 2.5 0.8% Agarose gel electrophoresis of dsRNA extracted from mycelial isolates of *T. harzianum* by CF-11 chromatography. Lanes are as follows:

M = Molecular weight marker (kb DNA Ladder)

1 = T7; 2 = KPNT; 3 = T32

4 = TH1C; 5 = TH3C; 6 = A006022

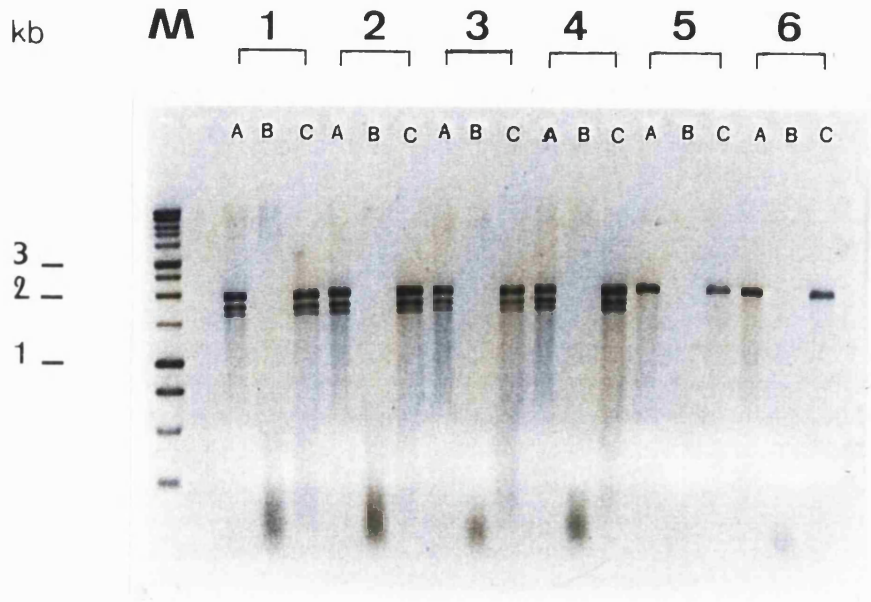


Fig. 2.6 Agarose gel electrophoresis of enzyme-digested components from *T. harzianum* six isolate :

Set 1 = T7; Set 2 = KPNT ; Set 3 = T32 ;

Set 4 = Th1c; Set 5 = Th3c ; Set 6 = A006022.

Lane M : Molecular weight marker (kb DNA ladder)

Lane A : dsRNAs digested with 0.5 µg/ml RNase A in 2 x SSC for 10 min at 37 °C.

Lane B : dsRNAs digested with 0.5 µg/ml RNase A in 0.1 x SSC for 10 min at 37 °C.

Lane C : dsRNAs digested with 1 unit DNase I in 40 mM Tris -HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$ for 10 min at 37°C.

2.4 DISCUSSION

Extrachromosomal genetic elements were detected in : *A. bisporus* isolates V95 and EU , *V. fungicola* (V7-3 ,The Netherlands) , *V. psalliotae* (V5-2, New Zealand) and *T. harzianum* isolates T7, KPNT, T32, Th1c, Th3 c, A006022.

These were identified as RNA molecules by their sensitivity to digestion with RNase A at low ionic strength and their insensitivity to DNase I. They were recognized as dsRNA molecules by their resistance to RNase A digestion in a high-ionic strength buffer, as well as by their specific binding to CF-11 cellulose in 16% ethanol, a chromatographic resin used to separate dsRNA from ssRNA and DNA (Franklin, 1966; Morris and Dodds, 1979). These are standard tests that have been used to characterize the dsRNA molecules of other fungi.

Columns of cellulose CF-11 were used to separate phage R17 RNA replicative intermediates from nucleic acids extracted from R17- infected *E. coli* (Franklin, 1966). The procedure exploits the differential solubility and interactions of ss and dsRNAs in the presence of various concentrations of ethanol. RNA is applied to pre-equilibrated columns in 35% ethanol, at which concentration soluble RNA passes through the column. Elution with 15% ethanol releases high molecular weight ss-ribosomal and viral RNA. RNA molecules with higher orders of secondary structure such as ds-viral RNA replicative intermediates are finally eluted with buffer solution. The exact nature of the interaction of dsRNA with the cellulose matrix is not understood, but it may involve hydrogen bonds between the hydroxyl groups in dsRNA and cellulose. RNA purification procedures that include a cellulose CF-11 step can remove trace amount of dsRNA from RNA preparation (Nicholson, 1996).

In previous studies, up to 10 major dsRNAs have been observed in diseased fruit bodies and mycelium of the cultivated mushroom *A. bisporus* (Marino, *et al.*, 1976; ; Ross, *et al.*, 1986 ; Deahl, *et al.*, 1987; Wach, *et al.*, 1987; Harmsen, *et al.*, 1989; Koons, *et al.*, 1989;). In the Netherlands, nine dsRNAs are associated with La

France disease : L1 (3.6 kb), L2 (3.0 kb), L3 (2.8 kb), L4 (2.7 kb), L5 (2.5 kb), M1 (1.6 kb), M2 (1.35 kb), S1 (0.86 kb) and S2 (0.78 kb) (Harmsen, *et al.*, 1989). Furthermore, Sonnenberg, *et al.*, (1995 a) found a complete correlation between viral disease symptoms and the presence of the major dsRNA L1 to L5 and M2. On rare occasions, M1, S1 and S2 were missing.

In diseased American mushrooms, Marino, *et al.*, (1976) described the presence of only six dsRNA, possibly corresponding to the L1 to L5 plus M2 segments described here. Wach, *et al.*,(1987) investigated American diseased fruit bodies in more detail and found a similar pattern. However, they also observed the pattern found by the Harmsen group (1989) in diseased material from the Netherlands. American diseased samples apparently show more variation in the dsRNA pattern. For instance, in *A. bisporus* strain Campbell M1, dsRNAs L6 and M1 were missing.

In this study there was evidence that the dsRNA pattern of *A. bisporus* isolate EU differed from isolate V95 (UK) by the presence of there extra bands. These were L6 (2.3 kb), M3 (1.0 kb), S2 (0.78 kb).

Occasionally, dsRNA has been detected in other cultivated mushrooms. For example, *Pleurotus ostreatus* var. *florida* with a decreased mycelial growth rate, contained seven dsRNA segments. The size of the largest dsRNA was 8 kb whereas the smaller dsRNAs were 2.4 kb(doublet), 2.1 kb, 1.9 kb (doublet) and 1.7 kb. The 1.7 kb dsRNA was present in minor amounts and often escaped detection. Isometric virus particles with diameters of 24 and 30 nm were found. Mycelium with a normal growth rate lacked dsRNA (Van der Lende, *et al.*, 1995).

Chen *et al.*, (1988) described the presence of a polyhedral dsRNA virus from the straw mushroom, *Volvariella volvacea* , in which one dsRNA element sized 3.2 kb was detected and encapsidated in isometric VLPs of about 35 nm in diameter.

Barroso and Labarere, (1990) demonstrated that dsRNA molecules purified from the vegetative mycelium of the basidiomycete *Agrocybe aegerita* could be separated by a sucrose density gradient into two different RNase-resistant complexes. The first complex was the encapsidated genome 6.2 kb of an isometric mycovirus. The second consisted of three naked dsRNA molecules (1.9-1.8-1.7 kb respectively) associated with large vesicles or mitochondria.

One of the commercial strains of the golden winter mushroom *Flammulina velutipes* Sing. (Enokitake) was found to contain two dsRNA elements sized 1.9 and 1.8 kb. These were detected in mycelium derived from a spontaneously brown-coloured fruit body. They were not detected in the normal strains or in fruiting - impaired degenerative isolates. The dsRNAs were not in the nuclear or mitochondrial fractions, but were located in the cytoplasmic fraction. The presence of virus-like particles of ca. 50 nm diameter associated with the dsRNA was confirmed by electron microscopic observation (Magae and Hayashi, 1999).

The ascomycete *Atkinsonella hypoxylon* (Peck) causes choke disease on several grasses. Three dsRNA were evident from *A. hypoxylon* isolate 2H. Their sizes were approximately 2.2 kb, 2.1 kb and 1.8 kb. The three segments had no significant similarity to each other, as determined by Northern blot analysis. Comparisons of the amino acid sequence deduced from dsRNA 1 revealed similarities with viral RdRp. Translation *in vitro* of full-length cDNA clones representing dsRNA 1 and 2 each yielded single major products of > 70 kDa. Based on properties of its dsRNA segments, the virus of *A. hypoxylon* isolate 2H fits into the *Partitiviridae* family (Oh and Hillman, 1995).

According to these results, the detection of dsRNA elements in *V. fungicola*, *V. psalliotae* and *T. harzianum* had not appeared in published surveys in which all tested isolates of a filamentous fungal species were found to harbour dsRNAs.

T. harzianum strains T7, KPNT, T32, Th1c (group I) from England and Northern Ireland contained 4 dsRNA segments. Group II Th3c and A006022 from Northern Ireland and Republic of Ireland contained 2 dsRNA segments. Initial results were consistent with the hypothesis that the dsRNA of these isolates from different geographical sources appeared to contain dsRNA of identical number and molecular weight and might be identical or have some sequence homology.

Tooley, *et al.*, (1989) showed that geographically divergent isolates of *Phytophthora infestans* contained a range of dsRNAs that had different affect, more than one-third of the Mexican isolates harboured VLPs, while these elements were absent in European isolates. Recent work by Fekete, *et al.*, (1995) reported that fifty-five geographically different strains of *Fusarium poae* were found to harbour dsRNA elements and encapsidated VLPs. There were great individual differences in dsRNA patterns of various strains.

The results revealed the presence of dsRNA in *A. bisporus* , *V. fungicola* , and *T. harzianum*. Furthur objectives are to study the effect that this dsRNA exerts on phenotypic expression and the relationship between virulence and presence of dsRNA in these fungi.

CHAPTER 3

Effect of dsRNA in fungi

CHAPTER 3 Effect of dsRNA in fungi

3.1 INTRODUCTION

In order to study the possible effects of dsRNA and dsRNA viruses on fungal phenotypes, it is necessary to obtain dsRNA containing and dsRNA-free isogenic lines. The techniques available for virus elimination include hyphal tip culture, chemotherapy and heat therapy (Nair, 1973).

Heat therapy is well established as a technique for the treatment of certain plant virus disorders (Nyland and Goheen, 1969). This approach has also been used to treat La France-diseased *Agaricus bisporus* cultures studied by Nair (1973). Eight virus-infected isolates of *A. bisporus* of various origins and three uninfected ones were incubated at 33°C for 1 to 6 weeks. The effect of heat treatment was assessed in terms of increased growth of the isolates *in vitro* and absence of virus particles. In his opinion, growth rate was not always a good measure of effective heat treatment. Treatment for at least 3 weeks at 33°C was recommended for virus elimination.

Van Zaayen, (1979) prepared both treated (3 weeks at 33°C) and untreated mycelia from nine of the most promising infected cultures as well as one uninfected culture. These mycelia were used as inocula in experiments to verify whether the treatment indeed freed the mycelial cultures from virus. The heat-treated derivatives of infected cultures were far from virus-free, as all three types of virus particles could often be extracted from the mushrooms. However, mushroom yields were sometimes increased by the treatment. In another experiment, a severely diseased culture with very slow growth was treated for 60 and 120 days, respectively, at 30°C, and a control of the same culture was kept continuously at 25°C. Again, mushroom yields were increased, but they were not back to normal and virus particles could be isolated from these mushrooms of rather poor quality. This mycelium obviously was not completely free of virus (Van Zaayen, 1979).

Chemical treatments have also been used to eliminate viruses and virus-like particles from eukaryotes. Cycloheximide-treated killer strains of *Saccharomyces cerevisae* lost the ability to inhibit other sensitive yeast strains (Fink and Styles, 1972). Later work by Young and Yagui (1978) found that loss has been attributed to the selective removal of a dsRNA segment associated with the ScV particle M in the cytoplasm of the killer strain of yeast.

Fulbright (1984) showed that dsRNA containing hypovirulent strains of *Endothia (Cryphonectria) parasitica* appeared morphologically normal and contained no dsRNA after cycloheximide treatment. However, certain hypovirulent genotypes were not cured of dsRNA by cycloheximide.

Later work by Melzer and Bidochka (1998) reported that each isolate of *Metarhizium anisopliae,* from fifteen colonies, each assumed to be derived from a single conidium, were segregated and grown for 14 days on potato dextrose agar (PDA). Conidiospores from these cultures were then transferred onto PDA containing cycloheximide (0.2g/ l PDA) and grown for 14 days. Mycelium taken from each colony perimeter was then transferred to PDA and grown for an additional 24 days. Over 100 strains were analyzed for dsRNA after attempts at curing and one such strain was recovered with complete loss of dsRNA.

Mycovirus are transmitted intracellularly during cell division, sporogenesis and cell fusion. As a result of these intracellular modes of transmission, the natural host ranges of mycoviruses are limited to individuals within the same or closely related vegetative compatibility groups (Anagnostakis,1982). Mycoviruses may be eliminated during sexual spore formation. Even though the yeast ssRNA and dsRNA viruses are effectively transmitted via ascospores, the mycoviruses infecting the ascomycete filamentous fungi are essentially eliminated during ascospore formation (Buck, 1986).

On the other hand, ssRNA and dsRNA mushroom viruses are transmitted efficiently *via* basidiospores (Van Zaayen, 1972). Mixed infections with two or more unrelated viruses are common, probably also as a consequence of the ways by which fungal viruses are transmitted in nature. There are apparently no structural interactions between these viruses since heterologous encapsidation has not been reported in mixed infections (Buck, 1986 and Ghabrial, 1994).

The infections due to mycoviruses are both latent and persistent. Latency benefits the host for survival, and persistence benefits the virus in the absence of infectivity (extracellular mode of transmission). Fungal viruses thus appear to have coevolved in concert with their hosts. To ensure their retention, some fungal viruses have evolved to confer some selective advantage upon their host (e.g. the killer phenotypes in yeasts and smuts). Nevertheless, mycoviruses persist and spread even without a selective advantage due to their efficient means of intracellular transmission (Ghabrial, 1998).

Although a large number of the viruses that infect plant pathogenic fungi have been reported to be avirulent, it is becoming increasingly clear that phenotypic consequences of harboring specific mycoviruses or certain dsRNA molecules can range from symptomless to severely debilitating, and from hypovirulence to hypervirulence (Ghabrial, 1994 ; Nuss and Koltin, 1990). Virus-induced diseases and virus-mediated attenuation of virulence in plant pathogenic fungi provide excellent opportunities for fundamental studies aimed at developing novel biological control measures.

The hypovirulence phenotype in the chestnut blight fungus *C. parasitica* is an excellent and well documented example of a mycoviral-induced phenotype that is currently being exploited for biological control (Choi and Nuss, 1992).

The debilitating disease of *Helminthosporium victoriae*, the causal agent of Victoria blight of oats, is an example of pathogenic effects of fungal viruses (Ghabrial, 1986), in which virulence can be modulated by the presence of virus-like dsRNA genetic elements. The diseased isolates B-1 and A-9 of *H. victoriae* contained dsRNA viruses, grown at a much slower rate than normal isolates, and produces irregular mycelial mats with little or no sporulation. The two isolates differ, however, in the outstanding disease symptoms considered typical of each isolate. Isolate B-1 was characterized by extensive collapse of aerial mycelium in young colonies grown on potato dextrose agar (PDA) medium. Scattered areas of lysed and collapse aerial mycelium were observed throughout the colony. Isolated A-9 was characterized by stunted colonies with sparse aerial mycelium and almost complete inhibition of colony expansion (Ghabrial, 1986).

The aims of this chapter were to examine the effects of the presence of the dsRNA and dsRNA-free in *A. bisporus* on morphology and growth rate. Also in this study *V. fungicola* isolate V7-3 and *T. harzianum* isolate T7 were cured of dsRNA by heat treatment and were compared with dsRNA- containing isolates with respect to fungal growth parameters, sporulation and virulence.

3.2 MATERIALS AND METHODS

3.2.1 Heat treatment

V. fungicola isolate V7-3 and *T. harzianum* isolate T7 cultures containing dsRNA were grown on 1% MA at 32° and 37°C for 4 weeks. The plates were observed every 2 weeks for visible changes in morphology or growth patterns and rates. Subsequently, 1 mm diameter plugs were taken from colony margins and transferred onto fresh 1% MA every 4 weeks for 6 months. After 6 months, these cultures were also grown in stationary liquid culture as follows: 20 ml of medium broth (2% malt extract, 2% mycological peptone) contained in a Petri dish was inoculated with two pieces of 6 mm-diameter mycelium plugs from heat-treated cultures and maintained at 25°C without agitation. Cultures were incubated for 2 weeks and harvested. These cultures were also extracted and dsRNA purified to compare with the control culture.

3.2.2 Influence of dsRNA on growth measurement

Radial growth rate was assessed on cellophane-covered 2% MA , that was dispensed in plastic Petri dishes (85 mm. diameter). To examine the effect of the dsRNA in *A. bisporus* (isolate V95 and EU) cultures were compared with dsRNA-free cultures. *V. fungicola* isolate V7-3 and *T. harzianum* isolate T7 cultures were compared with heat-treated cultures. Plugs of mycelia 6 mm were placed in the centre of 2% MA; 10 plates of each fungus were prepared. After incubation in the dark at 24-25 °C , the diameter of each colony was measured every day. Measurements were made along two axes intersecting at right angles , with these two measurements averaged for each plate.

Mycelial dry weight was also assessed at 20 days by collecting the fungal mat and dried completely at 42°C and weighed. Ten replicates per isolate were used. Differences between the mean diameter for each fungus were compared using a least significant difference t-test mean separation procedure.

Colony morphology was determined on 2% malt extract agar. Two types of colony morphologies were observed, appressed (A) and fluffy (F).

3.2.3 Influence of dsRNA on sporulation

To estimate the sporulation capacity of *V. fungicola* isolate V7-3 and *T. harzianum* isolate T7 cultures were compared with heat-treated culture. Plugs of 6 mm diameter were taken from the growing edge of cultures and transferred to fresh 2% MA. Total conidia production was determined after 14 days of growth at 25°C. Conidia were washed from the plates with 10 ml of 0.05% Tween 80 (polyxyethylene sorbitan monolaurate) to help disperse the spores. Appropriate dilutions of resuspended conidia were determined using a haemacytometer and the counts averaged from five replicate cultures with ten readings per culture. Differences between average number of conidia for each treatment were compared using a least significant difference (LSD) mean separation procedure.

3.2.4 Virulence assessment

Pathogenicity tests were conducted as described by Bonnen and Hopkins (1997). Briefly, conidial suspensions of *V. fungicola* (isolate V7-3 ; V7-3 heat treated) and *T. harzianum* (isolate T7 ; T7 heat treated) were prepared by washing 2 week-old cultures (2% MA) with distilled water. The conidia were rinsed once and resuspended in distilled water at a concentration of 10^5 conidia /ml. Whole caps (15 caps per treatment) from freshly harvested mushrooms were placed in moist chambers, drop inoculated (15µl = $1.5x10^3$ conidia/cap) with the conidial suspension of *V. fungicola* , *T. harzianum* and distilled water (control) and incubated at 22-25°C.

Disease progress was assessed each day for 5 days. The assessment categories were as follows:

0 = no symptoms

1 = faint rings around inoculation site.

2 = light brown rings around inoculation site

3 = dark brown rings at inoculation site

4 = dark brown rings, some sporulation and pitting of the cap tissue at inoculation site.

5 = the disease extends beyond inoculation site, severe pitting of the tissue and profuse sporulation.

Differences between average of symptom ratings for each treatment were compared using a least significant difference (LSD) t-test mean separation procedure (Bonnen and Hopkins, 1997).

3.3 RESULTS

3.3.1 Heat treatment

V. fungicola

V. fungicola isolate V7-3, previously shown to contain dsRNA segments was cultured at 32°C and 37°C. A culture was found to contain a sector with more aerial mycelia than the surrounding culture at 32°C after incubation for one month. The isolate at 32 °C was subcultured every month for six months from colony margins of fluffy white aerial mycelia sector. At 37°C the *V. fungicola* isolate V7-3 was very sensitive to this temperature and failed to grow in the first week of experiment. Thus it was only possible to analyse the 32°C treated culture for dsRNA by agarose-gel electrophoresis. No dsRNA was detected in cultures grown at 32°C for 6 months. Parallel isolations of the parent V7-3 culture contained the characteristic 1-kb to 2.3-kb dsRNA segments, but V7-3h (heat-treated) was dsRNA free (Fig 3.1; A).

T. harzianum

T. harzianum isolate T7, previously shown to contain dsRNA segments was cultured at 32°C and 37°C. Cultures treated at 32°C and 37°C were subcultured from colony margins every month for six months. The 37°C treated cultures were analyzed for dsRNA segments by agarose-gel electrophoresis. Some dsRNA were still detected after six months at 37°C, although, the yield of dsRNA after heat treatment at 37°C for six months was decreased by about six to seven times (1.5 μg of dsRNA / g fresh wt of mycelium) from that obtained at 25 °C (control; 9 μg of dsRNA / g fresh wt of mycelium). Isolate T7h (heat-treated) contained the characteristic 1.8-kb and 1.95-kb dsRNA segments. Parallel isolations of the parent isolate T7 culture contained the characteristic 1.65-kb to 2.0-kb dsRNA segments (Fig 3.1; B)

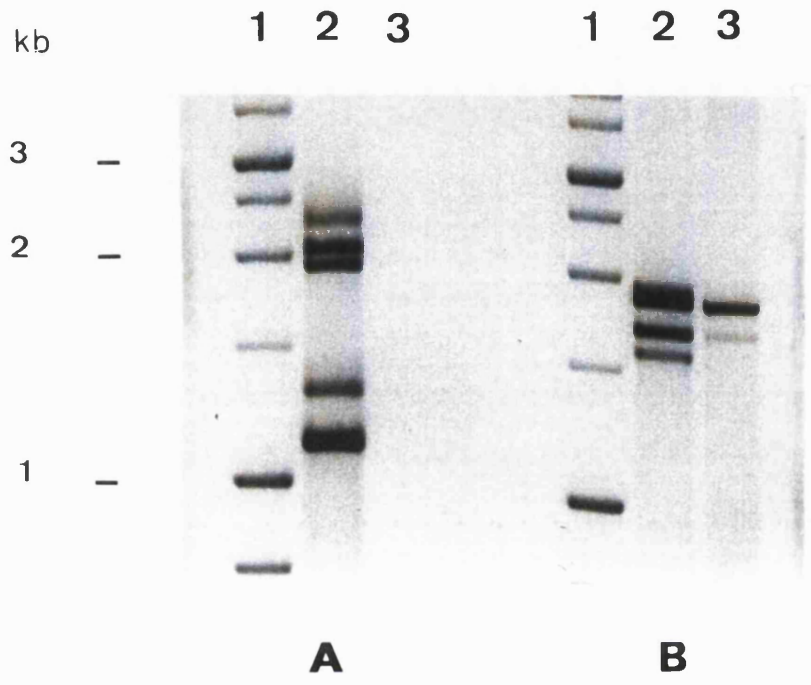


Fig 3.1 Agarose gel 1% analysis of dsRNA extracted from:

A: 1) Molecular weight marker (1kb DNA ladder)
2) *V. fungicola* isolate V7.3
3) *V. fungicola* isolate V7.3h- heat treated

B: 1) Molecular weight marker (1kb DNA ladder)
2) *T. harzianum* isolate T7
3) *T. harzianum* isolate T7h-heat treated

3.3.2 Influence of dsRNA on culture morphology

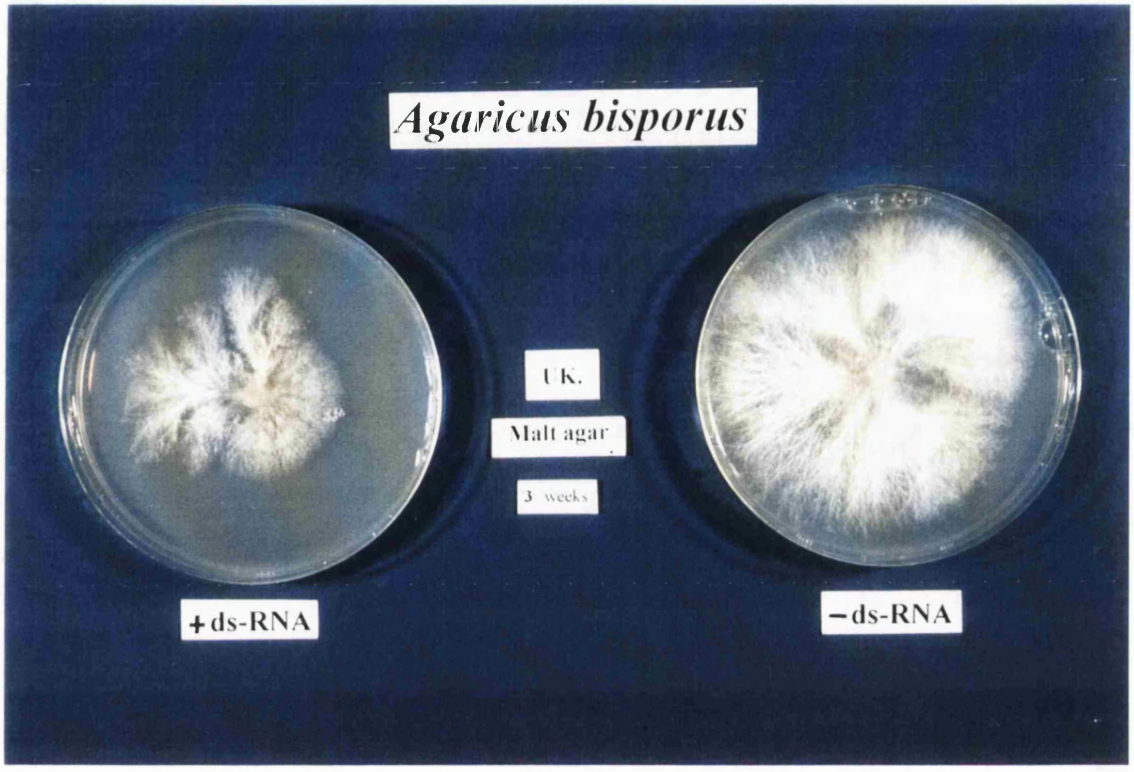
A. bisporus

Cultures of isolate V95 (+dsRNA) and EU (+dsRNA) grown on 2% MA showed a sparse, slow and appressed growth that was occasionally light brown instead of white colour. However, whereas some replicates grew very slowly, others showed moderately slow growth rates. An apparently healthy (dsRNA-free) *A. bisporus* isolate showed a vigorous and fluffy mycelial growth with strands radiating from the centre of the colony (Fig. 3.2 and 3.3).

Observation on colony growth rates showed that growth rates of the apparently healthy isolate were significantly higher than isolate V95 and EU (Fig. 3.4, Appendix II-1). When dry weight of the isolate were examined , mycelial dry weight was about 50 and 70 % lower in isolate V95 and EU , respectively, compared with " apparently healthy" (Fig. 3.5, Appendix II-1.1 and 1.2).

V. fungicola

A culture of *V. fungicola* isolate V7-3 was found to contain a large sector with more aerial mycelium than the surrounding culture during experimental heat treatment. This sector, which was stable upon subculture, was termed V7-3h to denote its heat treatment and fluffy appearance relative to V7-3. Morphological and physiological characteristics were examined to determine similarities and differences between V7-3 and V7-3h isogenic counterparts. Two types of colony morphology were noted for *V. fungicola* when grown on 2% MA (Fig. 3.6). Isolate V7-3 (+dsRNA) showed a colony habit that was appressed and showed a growth rate lower than V7-3h (Fig. 3.7). Isolate V7-3h colony morphology showed fluffy white aerial mycelia across the colony surface. There was a significant increase in growth rate in V7-3h compared to the V7-3. When dry weight of V7-3h and V7-3 was examined, mycelial weight was about 30% higher in V 7-3h (Fig 3.8, Appendix II-2.1).

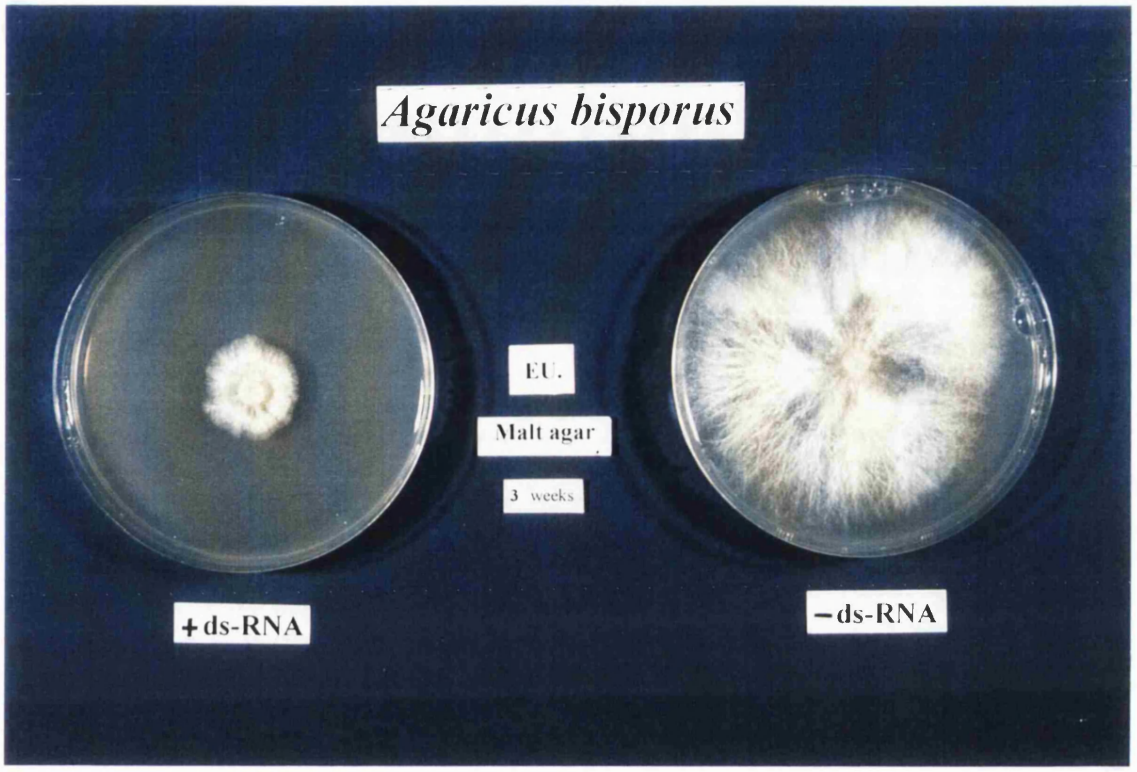


I II

Fig. 3.2 Cultural morphology of *A. bisporus* isolate V95 from UK on 2% MA media.

I) dsRNA-containing culture (+dsRNA).

II) dsRNA-free culture from apparently healthy (-dsRNA).



I II

Fig. 3.3 Cultural morphology of *A. bisporus* isolate EU on 2% MA media.

I) dsRNA-containing culture (+dsRNA).

II) dsRNA-free culture from apparently healthy (-dsRNA).

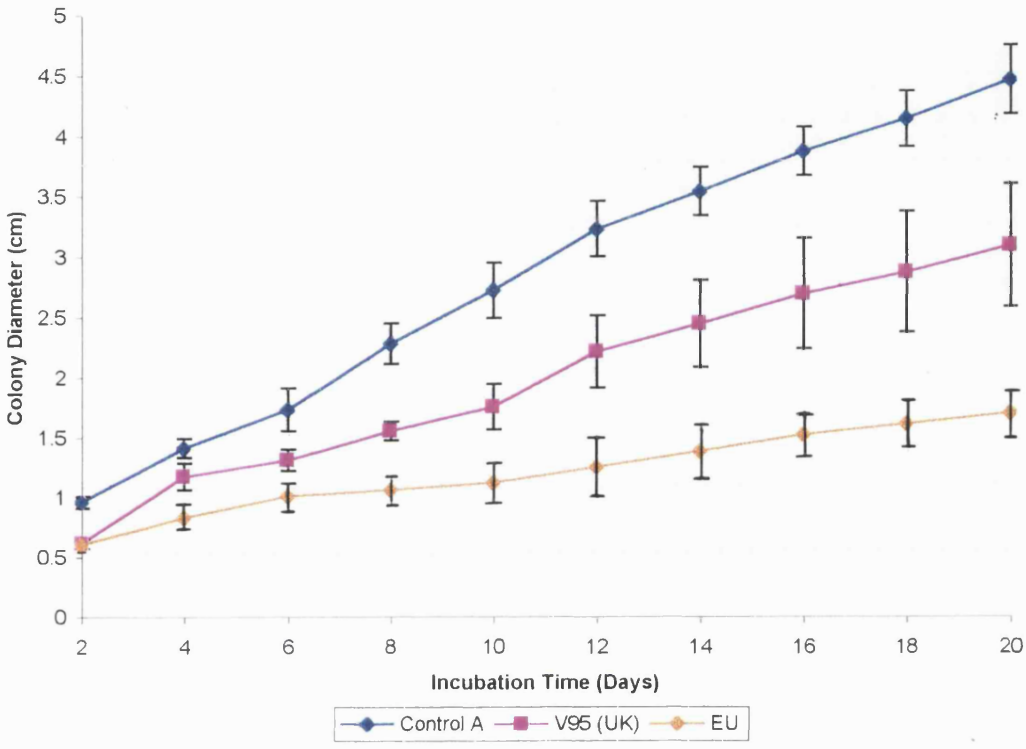


Fig. 3.4 Comparison of growth rate of dsRNA-free and dsRNA-containing cultures of *A. bisporus*. Cultures were grown on 2% MA media (ten replicates).

I) *A. bisporus* (dsRNA-free culture).

II) *A. bisporus* (isolate V95 from UK).

III) *A. bisporus* (isolate EU).

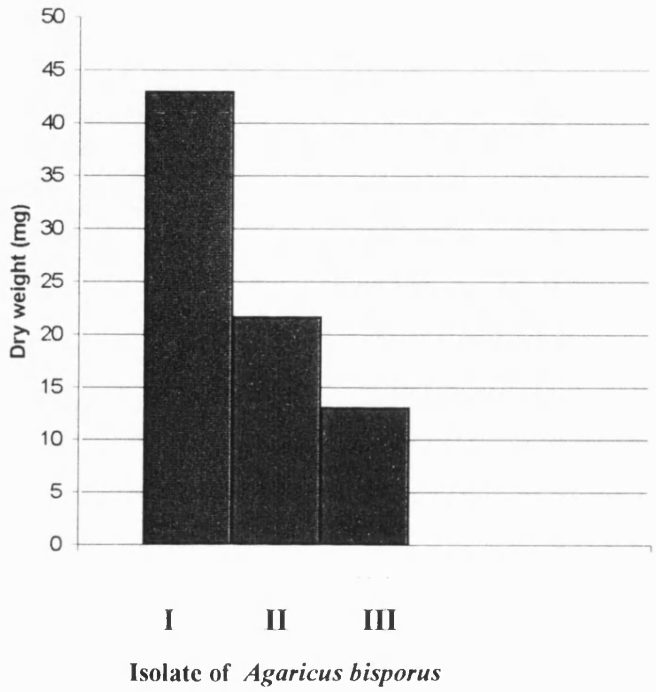
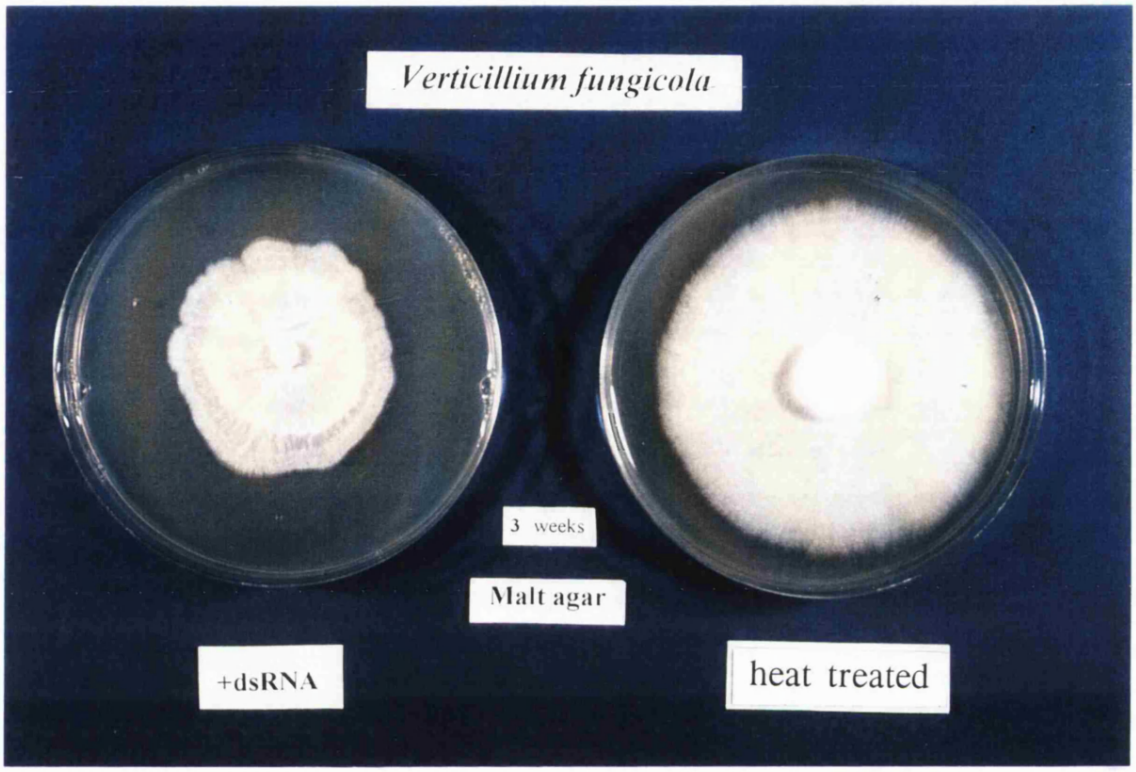


Fig. 3.5 Comparison of dry weight of dsRNA-free and dsRNA-containing cultures of *A. bisporus*. Cultures were grown on 2% MA media (ten replicates) Appendix II-1.1 and 1.2

I) *A. bisporus* (dsRNA-free culture).

II) *A. bisporus* (isolate V95 from UK).

III) *A. bisporus* (isolate EU).



I II

Fig. 3.6 Cultural morphology of *V. fungicola* isolate V7-3 on 2% MA media.

I) dsRNA-containing culture (+dsRNA).

II) heat treated culture.

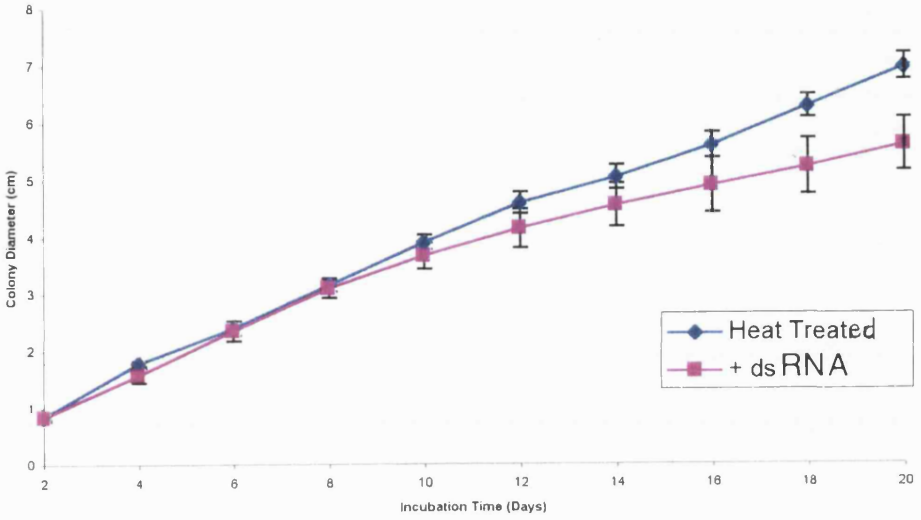


Fig. 3.7 Comparison of growth rate of dsRNA-heat treatment and dsRNA-containing culture .Cultures were grown on 2% MA media (ten replicates).

I) *V. fungicola* isolate V7-3h : dsRNA-heat treated culture.

II) *V. fungicola* isolate V7-3 : +dsRNA .

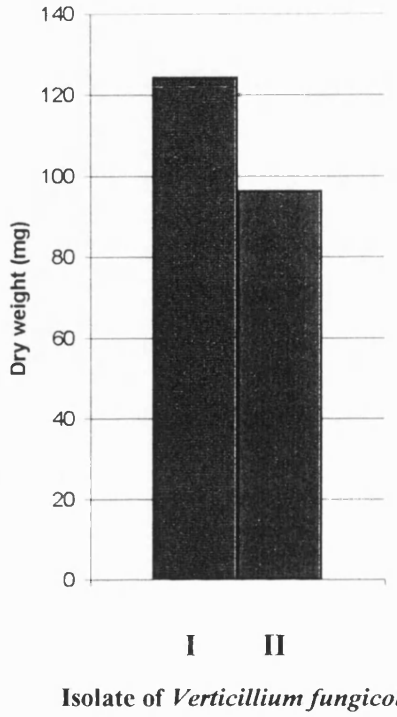


Fig. 3.8 Comparison of dry weight of dsRNA-heat treatment and dsRNA-containing culture. Cultures were grown on 2% MA media (ten replicates). Appendix II-2.1

I) *V. fungicola* isolate V7-3h ; dsRNA-heat treated culture.

II) *V. fungicola* isolate V7-3 ; +dsRNA.

T. harzianum

The morphology of *T. harzianum* isolate T7 (+dsRNA) was not significantly different from T7h (heat treated) on 2% MA (Fig. 3.9). Morphological and physiological characteristics were examined to determine similarities and differences between T7 and T7h isogenic counterparts.

There was no significant difference in growth rate in T7h compared to the T7. However, when the dry weights of T7h and T7 were examined, mycelial weight was significantly increased ca. 40% higher in T7h than T7 (Fig 3.10, Appendix II-2.2).

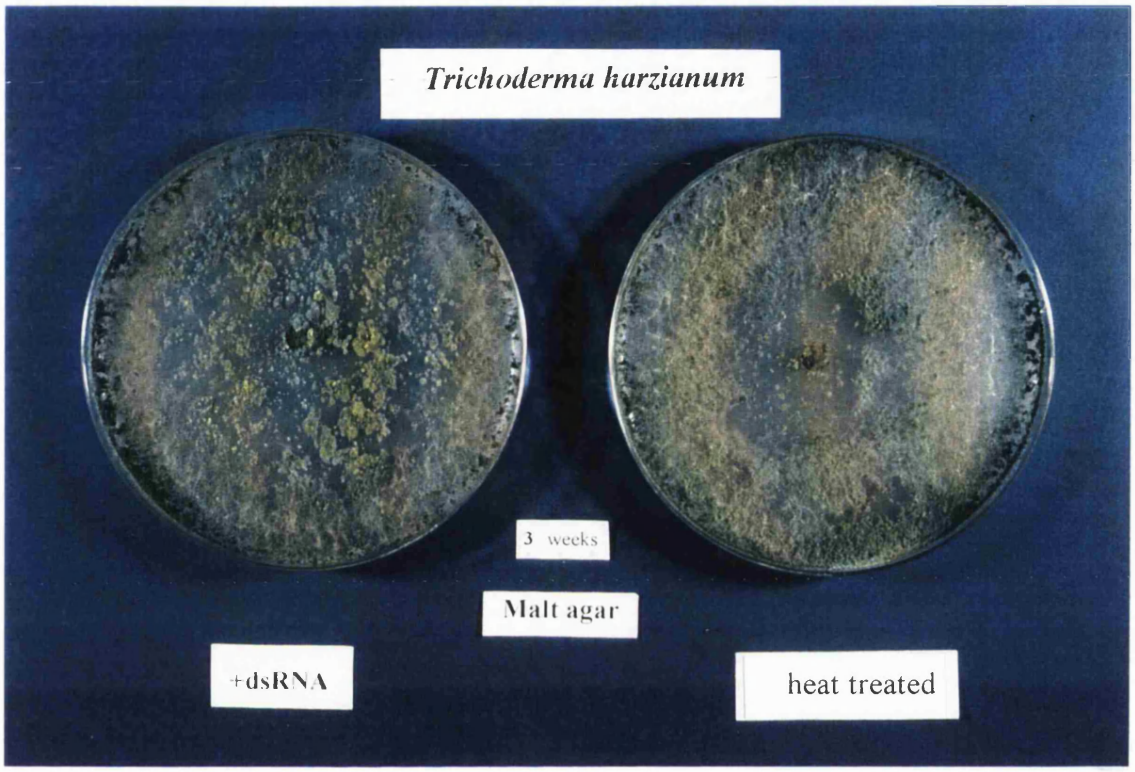
3.3.3 Influence of dsRNA on sporulation

V. fungicola

Morphological and physiological characteristics were examined to determine similarities and differences between V7-3 and V7-3h isogenic counterparts. To determine whether dsRNA affected sporulation, cultures were grown on 2% MA and was estimated by counting. As indicated in Figure 3.11, V7-3h (10.15×10^8 spore production / plate) was significantly increased by about 70 % in its ability to sporulate when compared to V7-3 (5.96×10^8 spore production / plate : Appendix II-3.1)

T. harzianum

Figure 3.12, shows sporulation of isolate T7h (4.40×10^9 spore production/ plate) was significantly increased ca. 63 % in its ability to sporulate when compared to T7 (2.69×10^9 spore production/ plate : Appendix II-3.2).



I II

Fig. 3.9 Cultural morphology of *T. harzianum* isolate T7 on 2% MA media.

I) dsRNA-containing culture (+dsRNA).

II) heat treated culture.

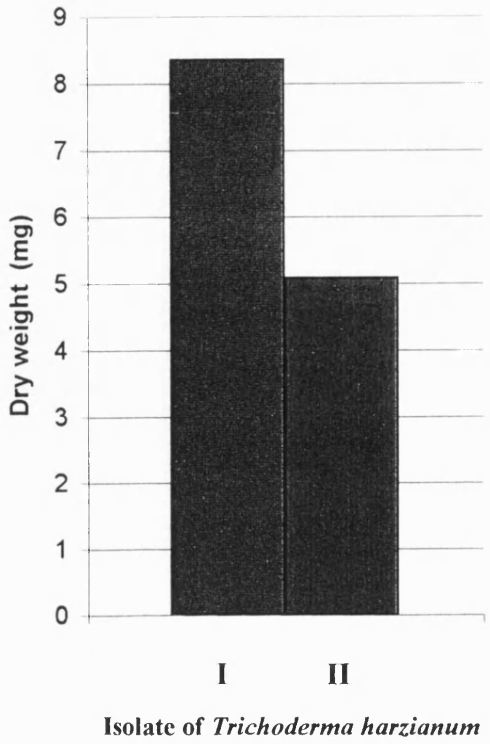


Fig. 3.10 Comparison of dry weight of dsRNA-heat treatment and dsRNA-containing culture. Cultures were grown on 2% MA media (ten replicates).
Appendix II-2.2

I) *T. harzianum* isolate T7h : dsRNA-heat treated culture.

II) *T. harzianum* isolate T7 : +dsRNA.

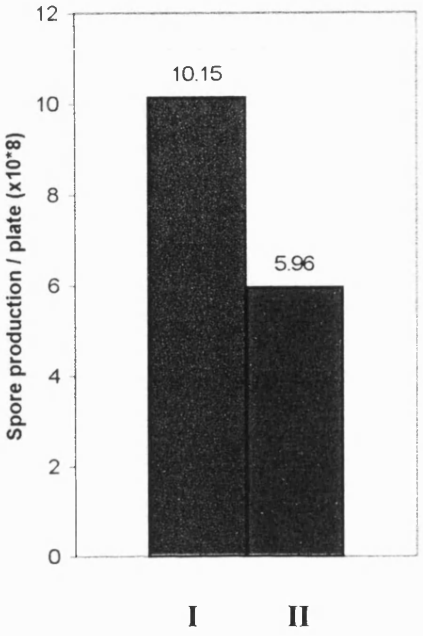


Fig. 3.11 Comparison of spore production of dsRNA-heat treatment and dsRNA-containing culture. Cultures were grown on 2% MA media (ten replicates). Appendix II-3.1

I) *V. fungicola* isolate V7-3h : dsRNA-heat treated culture.

II) *V. fungicola* isolate V7.3 :+dsRNA.

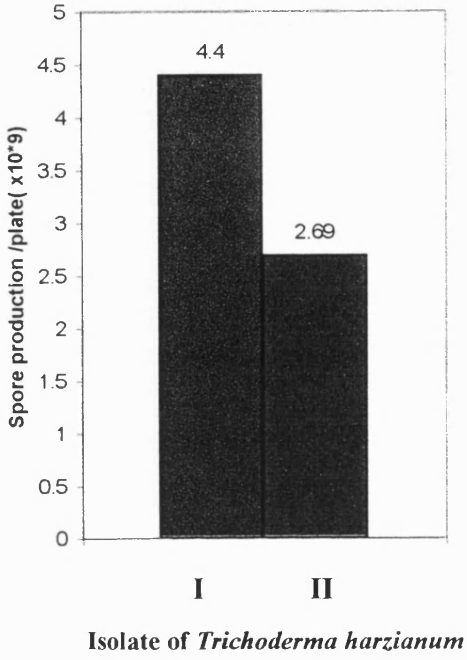


Fig. 3.12 Comparison of spore production of dsRNA-heat treatment and dsRNA-containing culture. Cultures were grown on 2% MA media (ten replicates). Appendix II-3.2

I) *T. harzianum* isolate T7h : dsRNA-heat treated culture.

II) *T. harzianum* isolate T7 : +dsRNA.

3.3.4 Influence of dsRNA on virulence

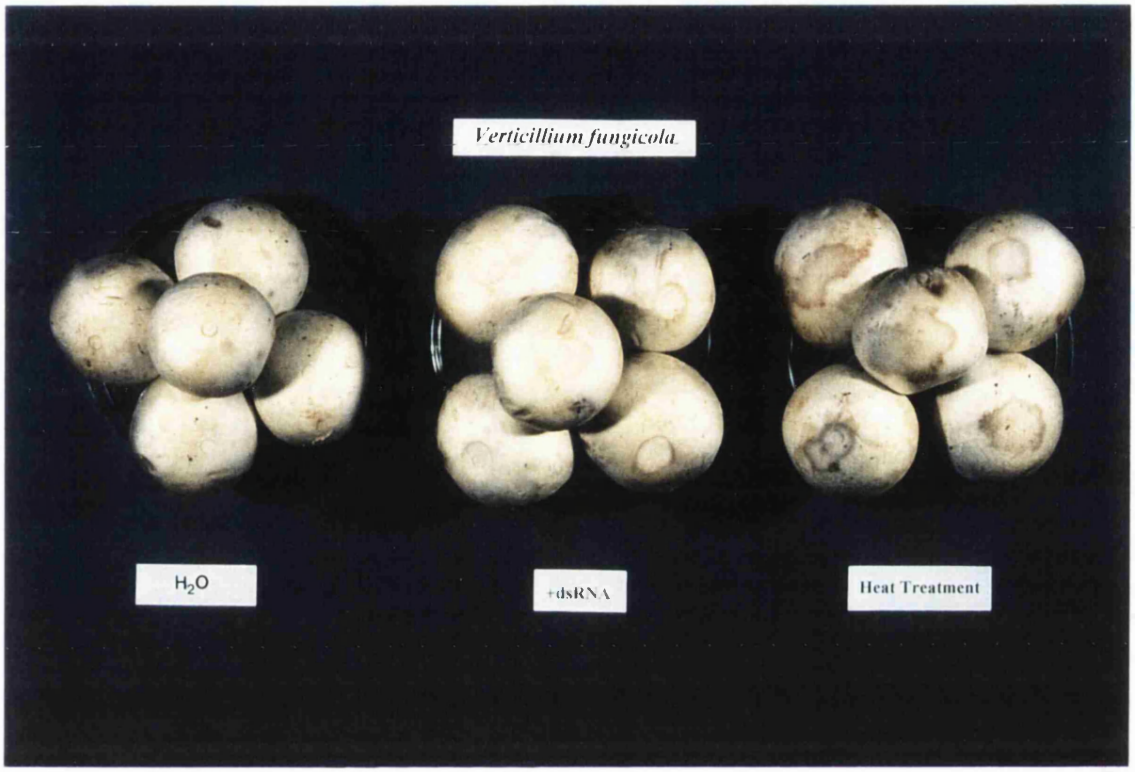
V. fungicola

Virulence of isolate V7-3h was examined by inoculation of mushroom sporophores. V7-3h produced significantly larger lesions on mushroom sporophores than either isolate V7-3 or the control (water) (Fig 3.13 : Appendix II-4.1). Visible symptoms of the disease were present within 3 days after inoculation. Surfaces of the basidiomes were covered with mycelium of *V. fungicola* and brown lesions had also developed. After 5 day, the basidiomes were grey-brown and severely infected in sporophores inoculated with V7-3h.

Isolate V7-3h was a typical case in which increased virulence was associated with the absence of dsRNA. Statistical t-test analysis of the assay indicated that virulence of V7-3h was significantly greater than the strain V7-3 (Table 3.1, appendix II-5.1).

T. harzianum

In the mushroom inoculations, dsRNA-heated treatment progeny isolate T7h were slightly more virulent than isolate T7. T7h produced lesions on mushroom sporophores the same size as isolate T7 , compared with the control (water) (Fig 3.14, Appendix II-4.2). Visible symptoms of the disease were present within 4 days after inoculation. A collapsed area was one which showed on surfaces of the basidiomes. Light brown lesions had also developed. After 5 day, the collapsed areas were more spread out. Statistical t-test analysis of the assay indicated that there was significant difference between isolate T7h and T7 (Table 3.1, Appendix II-5.2).



I II III

Fig. 3.13 Comparison of virulence test on mushroom for dsRNA-heat treatment and dsRNA-containing culture, after inoculation 4 days.

I) water (control).

II) *V. fungicola* isolate V7.3 : +dsRNA.

III) *V. fungicola* isolate V7.3h : dsRNA-heat treated culture.

Table 3.1 Comparison of disease progress on mushroom for *V. fungicola* isolate V7-3 ; heat treated and *T. harzianum* isolate T7 ; heat treated.

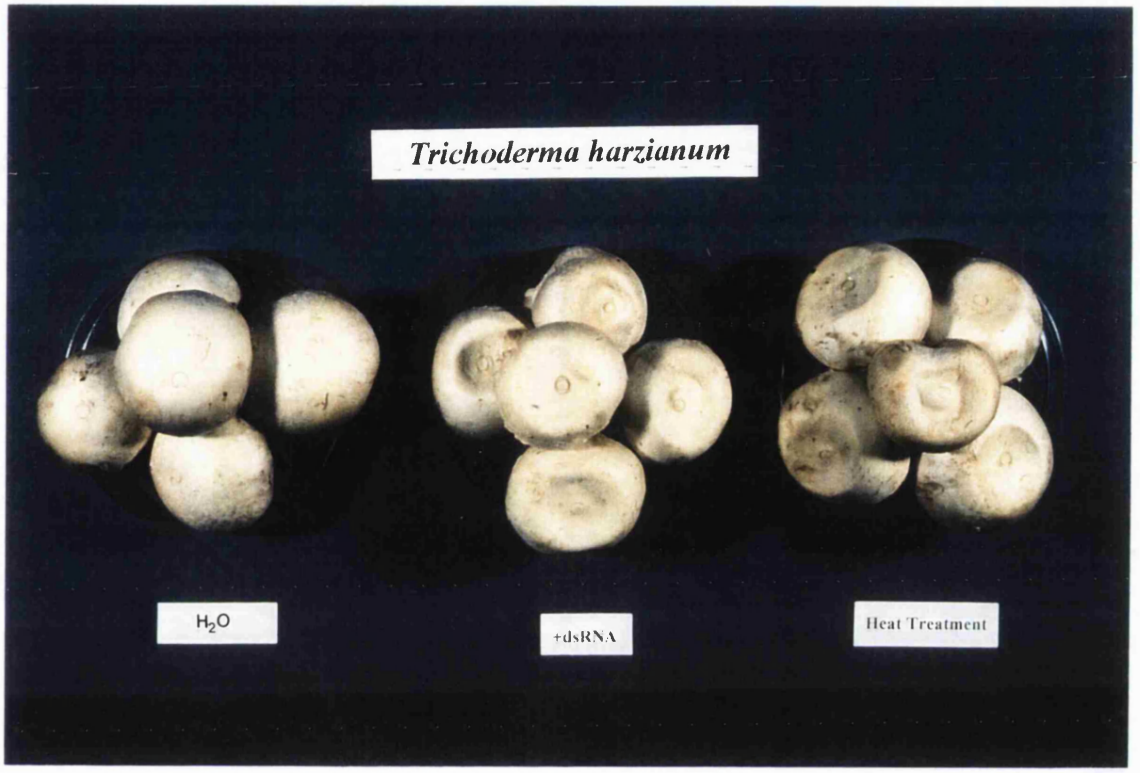
Culture		Disease progress	Lesion radius (mm)
V. fungicola (V7-3)	+dsRNA	1.8 [a]	12 [e]
V. fungicola (V7-3h)	heat treated	4.5 [b]	18 [f]
T. harzianum (T7)	+dsRNA	1.7 [c]	22 [g]
T. harzianum (T7h)	heat treated	2.4 [d]	24 [g]
Distilled water	(control)	0	0

-Disease progress based on a scale of 0-5 with ; 0 = no symptoms and 5 = severe symptoms.

-Means followed by the same letter are not significantly different at P= 0.05 using t-test minitab statistics package.

-Means are based on fifteen observations in each experiments.

-Appendix II-4 and II-5.



I II III

Fig. 3.14 Comparison of virulence test on mushroom for dsRNA-heat treatment and dsRNA-containing culture, after inoculation 4 days.

I) water (control)

II) *T. harzianum* isolate T7 :+dsRNA

III) *T. harzianum* isolate T7h : dsRNA-heat treated culture

3.4 DISCUSSION

V. fungicola isolate V7-3 was successfully cured of dsRNA by heat treatment at 32°C for six months. No dsRNA was detected in this isolate after prolonged subculture. The incubation temperature had a pronounced effect on the accumulation of dsRNA in diseased mycelium. Although heat-treatment at 37°C failed to eliminate all dsRNA segment in *T. harzianum* isolate T7, over a period of six months dsRNA levels in heat treated cultures were 5-6 times lower than controls.

Detroy, *et al.*, (1974) have shown that synthesis of viral dsRNA closely paralleled mycelial growth in *Penicillium stoloniferum*. This observation would expect dsRNA synthesis to proceed maximally near 24-25 °C in mycelium of *V. fungicola* and *T. harzianum*. Temperatures of 32 and 37°C were obviously outside the range of hyphal growth for most fungi including *V. fungicola* and *T. harzianum*.

Koons, *et al.*, (1989) reported that the temperature of growth had an effect on the accumulation of dsRNA in diseased mycelium of *A. bisporus*. The dsRNAs accumulated to their highest levels in cultures maintained at 21°C and 24°C. They were undetectable in cultures grown at 30 °C. Their findings agree with earlier reports of sporadic success in using heat therapy to cure disease cultures of VLPs (Van Zaayen, 1979).

Measurements of growth rate , dry weight production, sporulation and virulence, using representative cultures from *V. fungicola* isolate V7-3 and *T. harzianum* isolate T7 were compared with heat treated isolates. In V7-3 the presence of dsRNA was associated with a statistically significant difference in growth rate , dry weight and virulence when compared with V7-3h. However, T7 the presence of dsRNA was associated with a statistically significant reduction in dry weight and sporulation ; but growth rate, virulence, morphology was the same as T7h.

In *Lentinula edodes*, some strains that harbored dsRNA also did not exhibit any of the characteristic symptoms associated with dsRNA infection (Rytter, *et al.*, 1991). This was attributed to a "latent residential virus" condition, and a similar phenomenon could be occurring in *T. harzianum*.

Similar discrepancies were noted in earlier studies of *Endothia parasitica* strains containing dsRNA (Anagnostakis and Day, 1979). Therefore, although some of data appear to support a direct influence of dsRNA on selected fungal physiological parameters, they only provide putative correlative evidence because the genetic background of the isolates studied was different. Elimination of the dsRNA within the same genetic background has provided more convincing evidence for the effects of dsRNA in *V. fungicola* isolate V7-3.

Jeffries and Young, (1994) reported that the relation between *Verticillium* and *Agaricus* is of an invasive necrotrophic nature. The parasite hyphae penetrate the host hyphae, almost immediately accompanied by degeneration and death of the invaded cytoplasm. Studies of Dragt, *et al.*, (1996) found that several invasive necrotrophs penetrate host cell walls by a combined effect of wall-lytic enzymes and mechanical pressure. The latter is supported by the presence of appressoria and by the host cell wall that seems to be pressed inwards at the site of penetration. The thin wall of parasitized *A. bisporus* cells compared to the more electron-dense wall of the healthy cells supports the view that wall-lytic enzymes may play a role in the infection process.

Studies of Kalberer (1984) showing the presence of a possible serine protease in culture fluid of *V. fungicola* may further support this hypothesis, as cell walls of *A. bisporus* contain a relatively high amount of protein (Michalenko, *et al.*, 1976 and Novaes-Ledieu, and Garcia-Mendoza, 1981). Although secretion of hemicellulases appears to be a general process in fungi, no observations on *V. fungicola* are available so far to support this. However, if serine protease is also secreted inside the hyphae of *A. bisporus,* the severe nature of the pathogen may be due to intracellular proteolysis, leading to immediate cell death. The coenocytic nature of mycelium would allow rapid transport of protease to neighbouring cells, resulting in gradual lytic destruction of the cytoplasm and organelles.

Enebak, *et al.*, (1994) found the presence of dsRNA in *C. parasitica* significantly reduced the number of spores produced by isolate C-18 (+dsRNA). A 10-fold increase in the number of spores produced by dsRNA-free isolate C-18-23 over isolate C-18 was observed. The number of spores produce by isolate C-18-23 was similar to those produced by the dsRNA-free virulent control isolate Ep-155. In addition to reducing sporulation, the presence of dsRNA significantly reduced the production of laccase.

Reduced virulence is only one of a number of symptoms exhibited by strains of *C. parasitica* which harbour dsRNA genetic elements. Additional symptoms can include altered colony morphology, suppressed conidiation, reduced pigmentation and reduced levels of certain enzymatic activities, e.g. laccase and cellulase, and certain metabolites e.g. oxalate (Nuss and Koltin,1990). There is considerable variation in the level of virulence and the type of associated symptoms exhibited by different hypovirulent strains. Similarly, the dsRNAs associated with different hypovirulent strains vary considerably with respect to size, number of species, concentration and sequence homology. Similarly, dsRNAs linked to reduced aggressiveness in the Dutch elm disease pathogen *C. ulmi* (Roger, *et al.*, 1987) or hypovirulence in *C. parasitica* (Polashock and Hillman, 1994) are located in the mitochondria.

Melzer and Bidochka (1998) reported that after screening over 100 strains from several isolates of *Metarhizium anisopliae* they found phenotypic effects correlated to a particular dsRNA infection and, as such, this may not be a general phenomenon. The results revealed a difference in growth and conidiospore production (between the +dsRNA and the -dsRNA isogenic strains) of less than 2% and only on PDA supplemented with dodine. Furthermore, the dsRNA-free strain had elevated virulence when compared to its isogenic, dsRNA-containing, counterpart. Although the differences were statistically significant, the biological significance of dsRNA infection in these fungi remains questionable. Nonetheless, this single case shows the potential for the contribution of dsRNA infections on insect virulence in entomopathogenic fungi.

A better understanding of the V7-3h and T7h may afford clues to understanding dsRNA in fungus , virus transmission, and viral replication. Of particular interest is the identification of specific host factors that are involved in these processes.

This chapter results indicates that the presence dsRNAs can reduce growth in *A. bisporus, V. fungicola* and *T. harzianum*. In *V. fungicola* virulence can also be affected. Research on host-pathogen interactions and pathogenicity should consider the widespread occurrence of dsRNA in *V. fungicola* , *T. harzianum* and the possibility that the virulence may be affected.

CHAPTER 4

Purification of viruses and characterization of virus-like particle

CHAPTER 4 Purification of viruses and characterization of virus-like particle.

4.1 INTRODUCTION

Investigation of the commercially cultivated Basidiomycete *Agaricus bisporus* diseases has led to the discovery of fungal viruses (Hollings, 1962). Viruses have since been reported to occur in over 100 species from 73 genera of fungi, but only a small number of them have been isolated and characterized biophysically and biochemically.

Spherical to polyhedral and bacilliform viral particles have been reported in many edible fungi. In diseased *A. bisporus*, four kinds of polyhedral particles with 25, 29, 34 and 45 nm diameters and one kind of bacilliform particle, 19 x 50 nm have been found (Hollings, 1978). Spherical to polyhedral virus particles with diameters of 25, 30 and 39 nm, and occasionally rod-shaped particles about 15 nm wide and 700-900 nm long were observed on "Shiitake mushroom" *Lentinus edodes* (Ushiyama and Nakai, 1982).

Recently, spherical viral particles of 23 nm were shown to exist in oyster mushroom, *Pleurotus ostreatus* and *P. sapidus* in China (Liang, *et al.*, 1987). Two different oyster mushroom, *P. florida* and *P. ostreatus*, infected with viruses showed a slow growth on sawdust and rice straw substrates (Go, *et al.*, 1992 a). Symptoms observed by Go and coworkers included dense pinhead formation and the stipes of mushrooms were longer and slightly bent with smaller caps. The mushroom infected with viruses formed many branches on their stipes. Two different sizes of spherical-form VLPs were purified from unhealthy-looking mushrooms. It was found that abnormally grown *Pleurotus* spp. containing dsRNA showed typical symptoms of disease (Go, *et al.*, 1992 b). They could not isolate any detectable dsRNAs from healthy-looking mushroom isolates.

It was reported that dsRNA genomes were present in spherical to polyhedral viral particles such as the 39 nm particles from *L. edodes* (Ushiyama and Nakai, 1982) and the 23 nm particles from *P. sapidus* (Liang, *et al.*, 1987). Chen, *et al.*, (1988) reported 35 nm and a few 45 nm viral particles in preparation from *Volvariella volvacea* and that the VLPs were associated with one capsid polypeptide component having a molecular weight of 60 kDa.

Interest in an antiviral activity associated with two conidial fungi, *Penicillium stoloniferum* (Kleinschmidt, *et al.*, 1968) and *Penicillium funiculosum* (Lampson, *et al.*, 1967; Banks, *et al.*, 1968) led to early evidence for virus particles and to the discovery of viral dsRNA in fungi. Since these earlier studies, the evidence for viruses in fungi, mainly dsRNA-containing viruses, has grown substantially.

VLPs have been obtained from the ascomycetes fungus such as *Gaeumannomyces (Ophiobolus) graminis*, which is the cause of take-all disease of wheat and barley. When these crops are grown consecutively, the incidence of take all increases over three to five years and then decreases; a phenomenon known as take-all decline. Lapierre, *et al.*, (1970) reported that in France there was a correlation between take-all decline and the presence of isometric particles of 29 nm diameter in the fungus; isolates of "declined" fungus were weakly pathogenic, rarely produced perithecia, and in culture developed sectors and were difficult to maintain. Rawlinson, *et al.*, (1973) found in British isolates of the fungus two types of isometric particles with diameters of 35 nm and 27 nm, which sedimented homogeneously with coefficients of 148S and 110S respectively but were of heterogeneous densities between 1.29 and 1.37. Both types of isometric particle contained a single protein of 70 kDa. Frick and Lister (1978)reported that virus particles from American isolates had modal diameters of 35 nm, 39 nm and 41 to 42 nm ; one specific virus preparation had a polypeptide species of ca. 70 kDa. Considerable variation in the properties of viruses from different take-all disease *Gaeumannomyces (Ophiobolus) graminis* isolates has been observed, and some isolates have been found to be infected with as many as four different viruses. (Buck, 1986)

VLPs also occur in *Peziza ostracoderma*, which is a common contaminant of mushroom beds, and is probably the perfect stage of *Botrytis crystallina*. Some extracts of apothecia contain isometric particles 25 nm in diameter (Van Zaayen, *et al.*, 1970).

The dsRNA-containing virus particles described thus far from fungi are small, isometric particles characterized by a rather simple structure. They possess single-shelled or simple capsids, and particle diameters range from 20 to 50 nm. The distribution and complexity of their dsRNA genomes vary considerably, indicating that these viruses, despite their small size, simple structure, and characteristic dsRNA content, may be heterogeneous in evolutionary origin. Most of the dsRNA-containing fungal viruses examined thus far are serologically unrelated, and often serologically unrelated viruses exist within a given host strain. A strain of *Chalara elegans*, for example, has been shown to have at least five such viruses, each containing dsRNA (Bozarth and Goenaga, 1977).

The aims of this chapter were to examine and characterise VLPs in *A. bisporus* isolate V95, *Verticillium fungicola* isolate V7-3 and *Trichoderma harzianum* isolate T7 , and to describe the cytology of virus infecting fungi.

4.2 MATERIALS AND METHODS

4.2.1 Partial purification of virus like particles

To look for viral like particles (VLPs) in *V. fungicola* and *T. harzianum* (table 4.1) cultures were grown in stationary liquid culture (2% malt extract, 2% mycological peptone); purificaton of VLPs from *A. bisporus* was using material from fruit bodies. Frozen mycelia or fruit bodies (10 g) were homogenised in a blender with 40 ml of cold 100 mM potassium phosphate (KPO_4) pH 7. The homogenate was then extracted with 0.4 vol chloroform , centrifuged at 10,000 g for 25 min at 4°C and the upper aqueous phase transferred to a 100 ml flask and adjusted to 0.5 M. NaCl , 10 % PEG (6,000 M.W.) and stirred overnight at 4°C. The precipitate was collected by centrifugation at 15,000 g for 45 min at 4°C and resuspended in 1 ml of 20 mM KPO_4 pH 7 overnight. The resuspended pellet was clarified by centrifugation at 10,000 g for 10 min at 4°C and the supernatant retained (modified from Gillinds, *et al.*, 1993 and Howitt, *et al.*, 1995). Samples were used directly or stored at 4°C for 1-2 days until examined by electron microscopy. The UV absorption spectrum of each sample was determined with a spectrophotometer over the range of 200 to 350 nm.

Table 4.1 List of *A. bisporus*, *V. fungicola* and *T. harzianum* for purified VLPs.

Culture		dsRNA	Source
A. bisporus	isolate V95	+dsRNA	Fruit body
A. bisporus	Apparently healthy	-dsRNA	Fruit body
V. fungicola	isolate V7-3	+dsRNA	Mycelia
V. fungicola	isolate V61-7	-dsRNA	Mycelia
T. harzianum	isolate T7	+dsRNA	Mycelia
T. harzianum	isolate T7 reduced	-dsRNA	Mycelia

Flow diagram showing procedure for isolation of virus like particles from *A. bisporus*, *V. fungicola* and *T. harzianum*.

mycelia or fruit bodies

⇩

❶ Add to 1 part of mycelium + 4 parts of cold 0.1 M potassium phosphate pH 7

⇩

❷ Homogenise 5 min, then extracted with 0.4 vol/ volume chloroform

⇩

centrifuge 10,000 g, 25 min at 4 °C

⇩

❸ Buffer phase removed and adjusted to 0.5 M NaCl and 10 % PEG (mol wt 6,000)

stir overnight at 4°C

⇩

centrifuge 15,000 g , 45 min at 4 °C

⇩

❹ Resuspend the pellet in 1 ml 0.02 M potassium phosphate pH 7 overnight at 4 °C

⇩

centrifuge 10,000 g, 10 min at 4°C

⇩

❁ ❁ supernatant contains the virus ❁ ❁

⇩

- UV absorption
- Electron microscopic examination
- Analysis of viral protein

4.2.2 Electron microscopy

Negative staining of isolated VLPs

A 300 mesh pioloform coated grid was placed onto a 20 µl , drop of the partially purified sample and mixed in the ratio of 1:1 with the negative stain (2% potassium phosphotungstic acid pH 6.7) for 90 sec : excess stain was removed with filter paper. The air dried grids were examined for VLPs using a transmission electron microscope at 80 kV (Jeol JEM 1200EX). At least 200 particles were measured and photographed.

Ultrathin sectioning of mycelium

The mycelium of *A. bisporus* isolate V95, *V. fungicola* isolate V7-3 and *T. harzianum* isolate T7 were obtained from cultures grown on solid medium 2% MA. Specimens (2x2x4 mm) were fixed in 2% glutaraldehyde in 1% acrolein with 100 mM PIPES buffer pH 6.8 under vacuum for 10 min before fixed overnight. After washing in 150 mM PIPES buffer 3 times during 30 min, the specimens were post-fixed for 1 hr with 1% osmium tetraoxide in 150 mM PIPES buffer, and subsequently washed in distilled water three times during 30 min. After dehydration with a graded acetone series (50 , 70 , 80 , 90, and 100% v/v) for 25 min with each solution, these were infiltrated with Spurr's resin overnight (Spurr, 1969). Polymerization was performed at 70 °C for 8 hr.

Resin blocks were hand-trimmed and faced-off with a glass knife. Semi-thin sections (approx. 0.5 μm) for light microscopy were cut and stained with a " Epoxy Tissue Stain " (E.M.S; Fort Washington, U.S.A.) to identify tissue suitable for electron microscopy. Ultrathin sections were cut on a Reichert Om U3 Ultramicrotome (Leica Microscopy and Scientific Instruments Group, Heerbrugg, Switzerland) using a diamond knife and collected on copper grids (Agar Scientific Ltd, Stansted, UK : slot grids were previously coated with films of 0.4% w/v piolofrom in chloroform).

The sections were stained by the double lead stain technique (Daddow, 1983). Briefly, all grids were stained together in closed Petri dishes where drops of stains were pipetted on to clean parafilm squares within the dish. A carbon dioxide free environment for lead staining was achieved by placing sodium hydroxide pellets around the parafilm squares in the Petri dish. Each grid was placed on a drop of lead citrate stain for 2 min, rinsed and dried carefully prior to being placed on a drop of 6% uranyl acetate for 15 min. After several rinses in double-distilled water, each grid was again stained with lead citrate for 5 min, rinsed and dried before viewing in the electron microscope. Sections were examined with transmission electron microscope (Jeol JEM 1200EX) operating at 80 or 100 kV.

4.2.3 SDS-Polyacrylamide gel electrophoresis

Partially purified preparation of VLPs were analyzed on SDS-polyacrylamide gels using the discontinuous system of Laemmli (1970). To determined the molecular weight of virus-associated proteins, a 5% stacking gel with 10% resolving gel was used . Methods for gel and running buffer preparations followed that described by Sambrook, *et al.*,(1989).

Purified VLPs were solubilized in SDS-sample buffer. These samples were boiled for 5 min then directly applied on the gels. Electrophoresis was carried out with a constant 20 mA current using a "Mini-Protean II" unit (Bio-Rad) until the bromophenol blue migrated to the bottom of gel. Electrophoresed gels were stained with 0.1% of Coomassie Blue R-250 in solution of 50% methanol and 10% acetic acid for 1 hr then destained overnight in destaining solution (25% methanol, 7.5% acetic acid). Gels were calibrated with a low range molecular weight standards marker (BIO-RAD). The molecular weight of purified VLPs was obtained from electrophoretic migration relative to standards proteins which included rabbit muscle phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa) , soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.4 kDa). Gels were viewed on a light box and the position of the bands recorded by photography.

4.3 RESULTS

4.3.1 Partial purification

The ultraviolet absorption spectrum of partially purified VLPs from the *A. bisporus* isolate V95 preparation showed a maximum at 260 nm and a minimum at 245 nm, and the A_{260}/A_{280} ratio was 1.19. The *V. fungicola* isolate V7-3 preparation showed a maximum at 260 nm and a minimum at 240 nm, and the A_{260}/A_{280} ratio was 1.53. The *T. harzianum* isolate T7 preparation showed a maximum at 260 nm and a minimum at 235 nm, and the A_{260}/A_{280} ratio was 1.80. The results of all samples exhibited an ultraviolet absorption typical of nucleoprotein (Fig. 4.1).

4.3.2 Observation of virus like particles

Electron microscopic examination of the partially purified preparations of *A. bisporus* isolate V95, *V. fungicola* isolate V7-3 and *T. harzianum* isolate T7 revealed the presence of VLPs in the fruit bodies and mycelium of the strains examined. All of the VLPs were isometric and diameter average from 200 virus particles.

A. bisporus isolate V95, was observed mainly measuring 25 nm, but a few 34 nm particles were detected (Fig. 4.2). In the case of *V. fungicola,* isolate V7-3 contained isometric VLPs with a diameter of about 20 nm (Fig. 4.3). *T. harzianum* isolate T7 contained isometric VLPs with a diameter about 25 nm (Fig. 4.4).

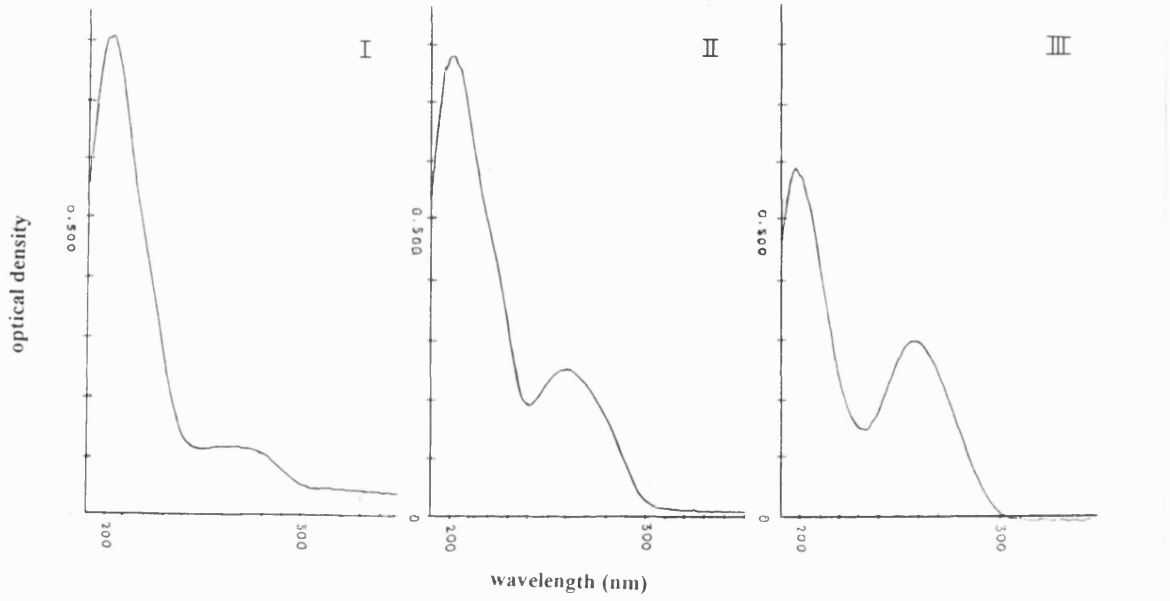


Fig. 4.1 UV absorption spectrum over the range of 200 to 350 nm of partially purified VLPs preparation.

I) *A. bisporus* isolate V95

II) *V. fungicola* isolate V7-3

III) *T. harzianum* isolate T7

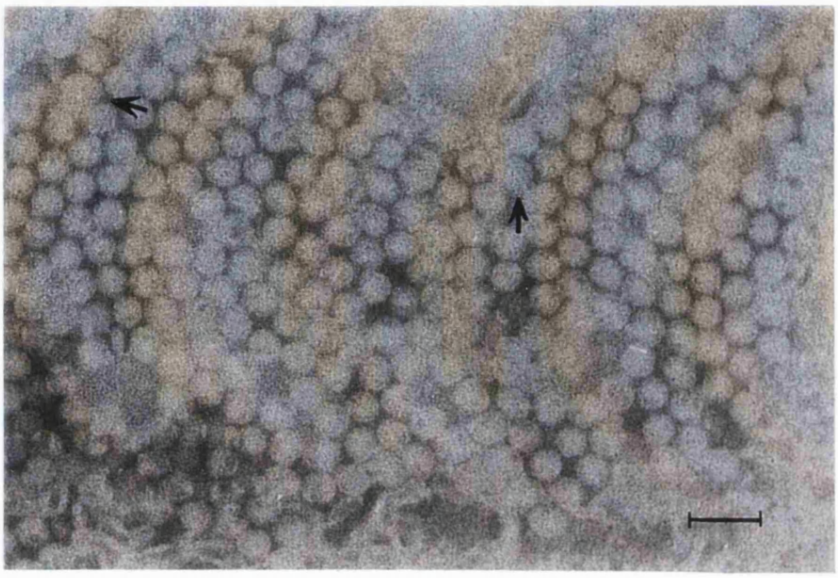


Fig. 4.2 Transmission electron micrographs of VLPs in partially purified preparations from fruit bodies of *A. bisporus* isolate V95 UK, negatively stained with 2% phosphotungstic acid pH 6.7. Isometric particles 25 nm in diameter, rare 34 nm particles (arrow). The bars marker represent 50 nm.

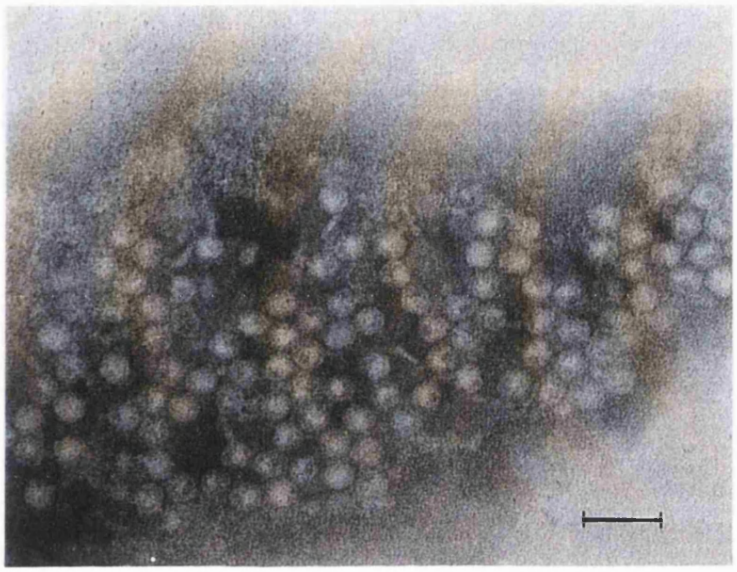


Fig. 4.3 Transmission electron micrographs of VLPs in partially purified preparations from mycelia of *V. fungicola* isolate V7-3,
negatively stained with 2% phosphotungstic acid pH 6.7.
Isometric particles about 20 nm in diameter.
Some VLPs are partially penetrated by stain. The bars marker represent 50 nm.

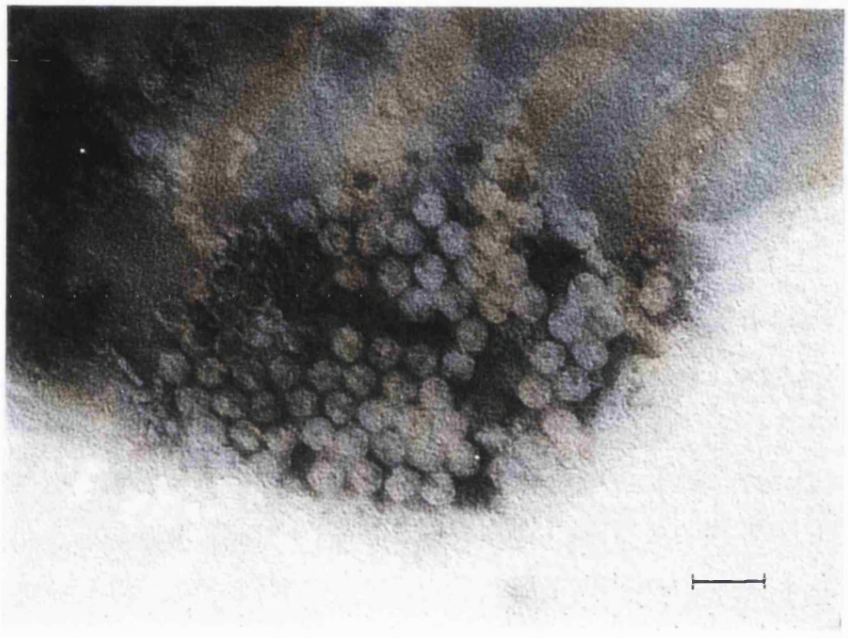


Fig. 4.4 Transmission electron micrographs of VLPs in partially purified preparations from mycelia of *T. harzianum* isolate T7, negatively stained with 2% phosphotungstic acid pH 6.7. Isometric particles about 25 nm in diameter. The bars marker represent 50 nm.

4.3.3 Intracellular appearance of VLPs

To detect the presence of intracellular VLPs, ultrathin sections of *A. bisporus* isolate V95, *V. fungicola* isolate V7-3 and *T. harzianum* isolate T7 mycelium were prepared and examined by electron microscopy. Mycelia ultrathin sections of *A. bisporus* isolate V95 showed the presence of VLPs of about 25 nm and 34 nm in diameter (Fig. 4.5), which were similar to those found in negatively stained preparations. Most particles were observed in the cytoplasm , but intranuclear particles were not observed.

Isometric VLPs of about 20 nm in diameter were observed in mycelium ultrathin sections of *V. fungicola* isolate V7-3. There particles were generally located in the cytoplasm or formed small clusters within membranous cytoplasmic structures (Fig. 4.6). VLPs were not observed inside the mitochondria or nuclei.

The visualization of *T. harzianum* isolate T7 mycelium ultrathin sections showed the presence of isometric particles of approximately 25 nm in diameter were in positively stained preparations in the cytoplasm of mycelium (Fig 4.7, A and B). The particles were darkly stained and were clustered throughout the cytoplasm (Fig 4.8). No virus particles were associated with nuclei, mitochondria or other organelles.

Occasionally in some mycelium sections, thread-like structures that had the typical appearance of nucleic acid were observed. This appearance to be attached externally to many capsids. These structures might be RNA-protein complexes (Fig. 4.9 A, B). In most VLP-containing cells some degeneration was observed (Fig. 4.10).

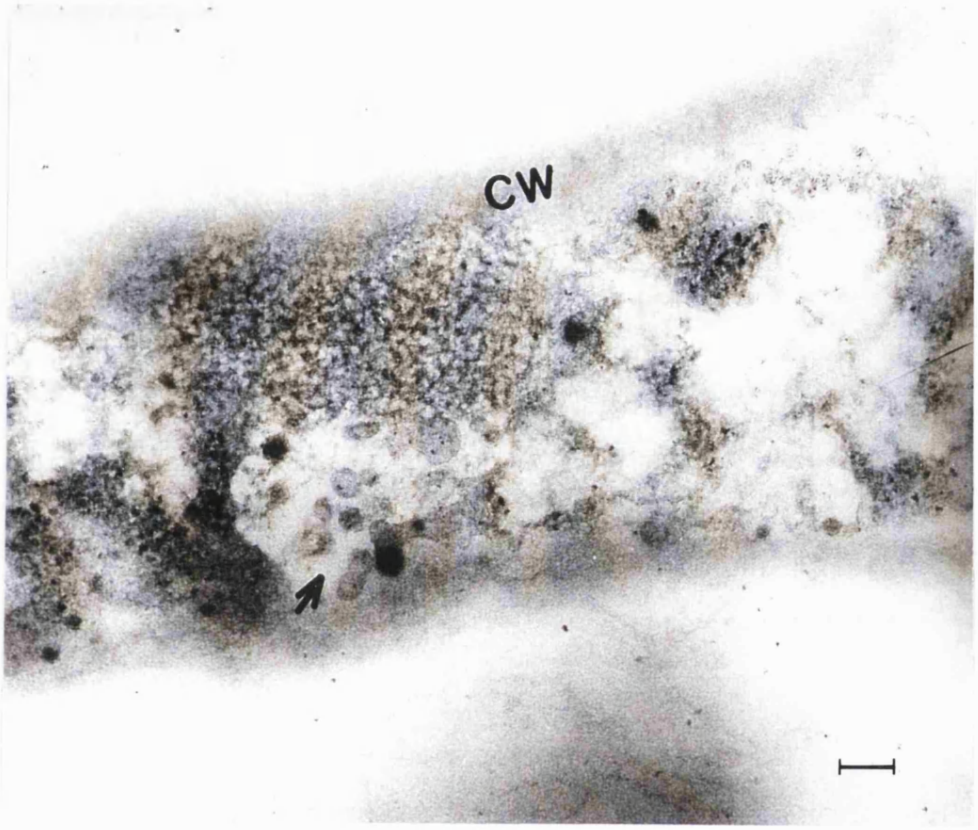


Fig. 4.5 Ultrathin section of virus-infected *A. bisporus* isolate V95 mycelium containing VLPs. The arrows indicate the position of VLPs, CW = cell wall. The bar marker represents 100 nm.

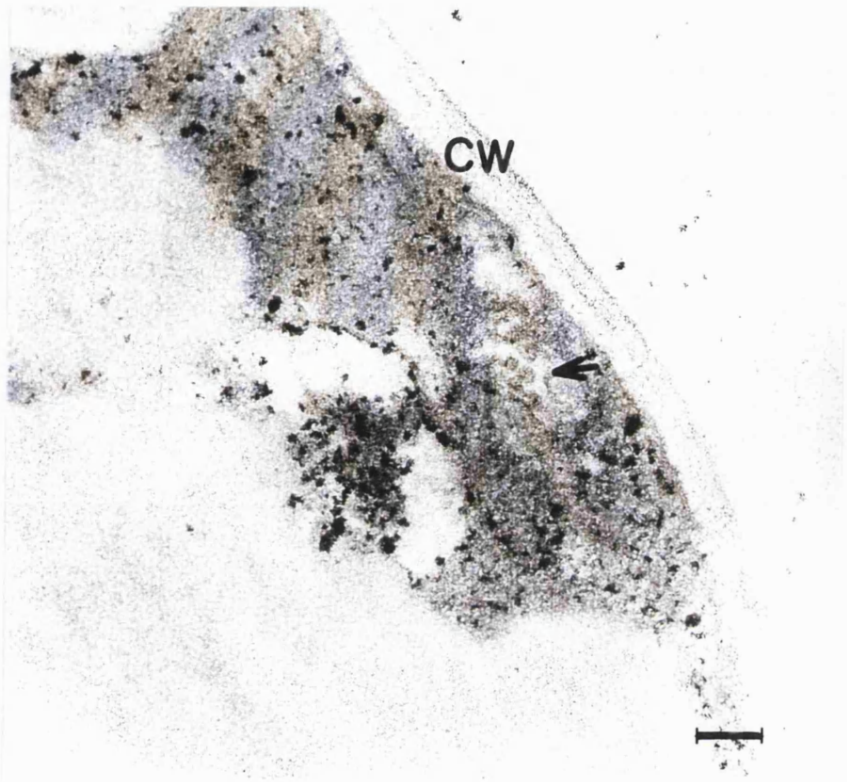


Fig. 4.6 Ultrathin section of virus-infected *V. fungicola* isolate V7-3 mycelium containing VLPs. The arrows indicate the position of VLPs, CW = cell wall. The bar marker represents 100 nm.

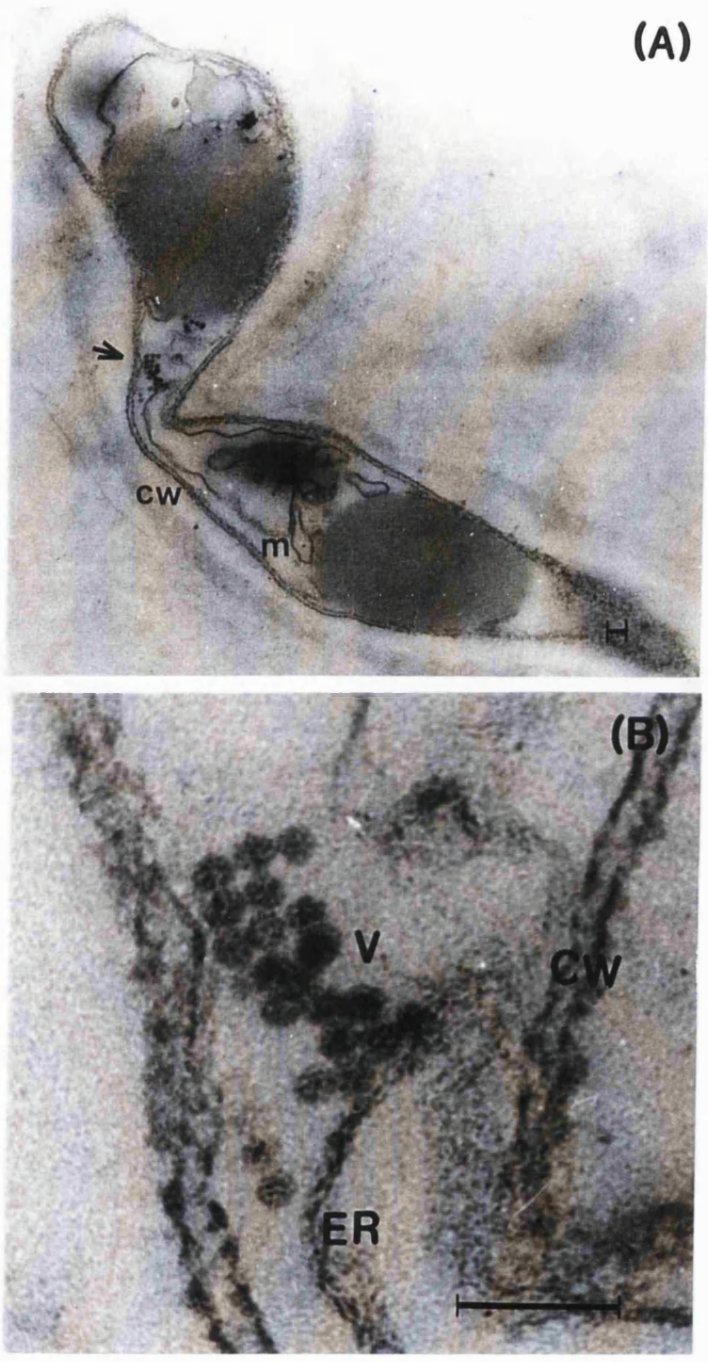


Fig. 4.7 Ultrathin section of virus-infected *T. harzianum* isolate T7 mycelium containing VLPs. The arrows and V that indicate the position of VLPs. CW = cell wall, ER= endoplasmic reticulum, M = mitochondria. The bar marker in **A** and **B** represents 100 nm.

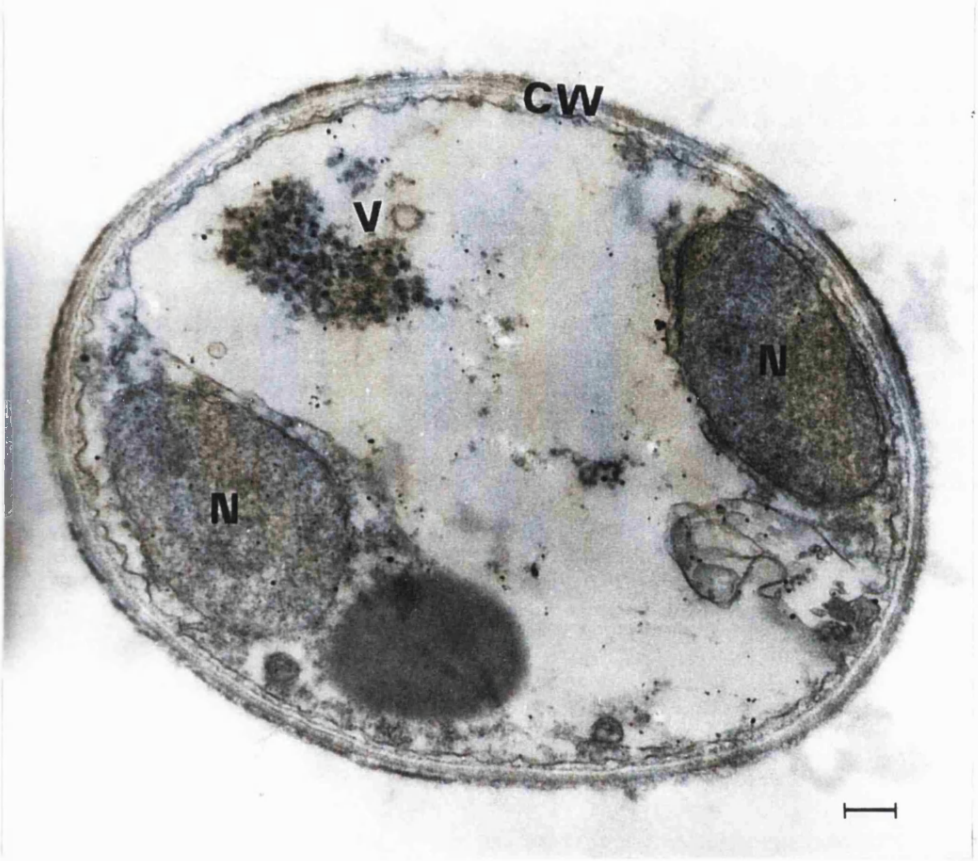


Fig. 4.8 Ultrathin section of virus-infected *T. harzianum* isolate T7 mycelium containing VLPs. V= indicate the position of VLPs.
CW = cell wall, N = nucleus. The bar marker in represents 200 nm.

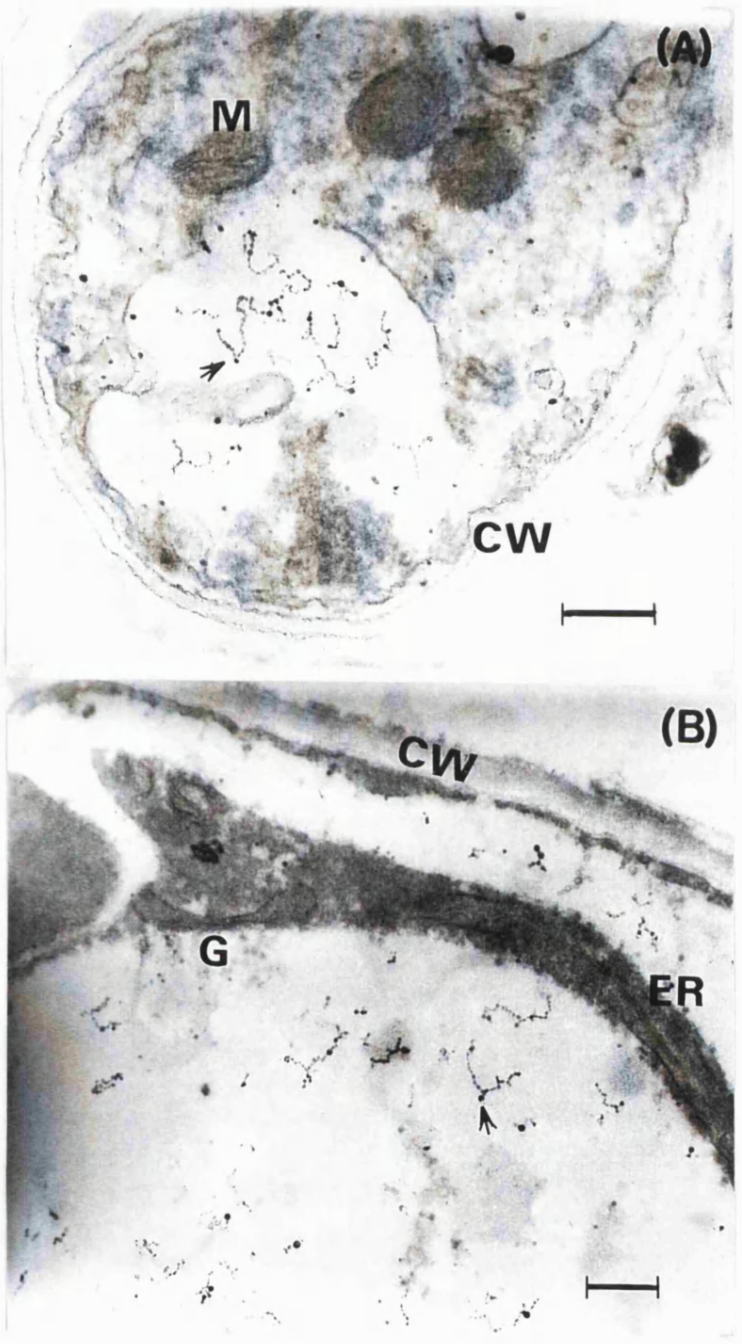


Fig. 4.9 Ultrathin section of virus-infected *T. harzianum* isolate T7 mycelium containing VLPs. The arrows indicate the position of VLPs.
CW = cell wall, ER= endoplasmic reticulum, M = mitochondria.
G = Golgi equivalents. The bar marker in **A** and **B** represents 200 nm.

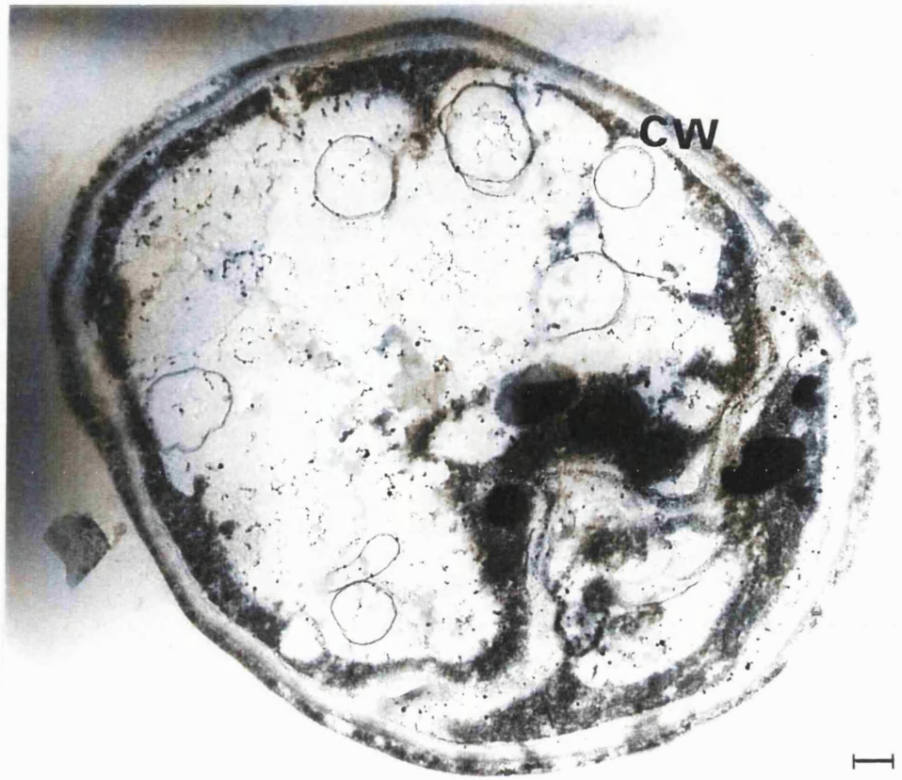


Fig. 4.10 Ultrathin section of virus-infected *T. harzianum* isolate T7 mycelium containing VLPs show degree of cellular degeneration.
CW = cell wall. The bar marker in represents 200 nm.

4.3.4 Viral protein

Following analysis of viral proteins, two major polypeptides of approximately 23 and 63 kDa were detected by SDS-PAGE in the purified VLPs of *A. bisporus* isolate V95 (Fig 4.11, lane 2). In addition, the healthy sporophores contained a polypeptide of approximately 61 kDa (Fig 4.11, lane 1).

Following electrophoresis of the purified VLPs of *V. fungicola* isolate V7-3 on a 10% polyacrylamide gel, the SDS-dissociated virus particles showed a single major band with molecular weight of 36 kDa (Fig. 4.12 lane 2). This protein is most likely the capsid protein.

Electrophoresis of the purified VLPs of *T. harzianum* isolate T7 by SDS-PAGE resolved into two major proteins with molecular weights of ca. 55 and 61 kDa (Fig. 4.13 lane 2).

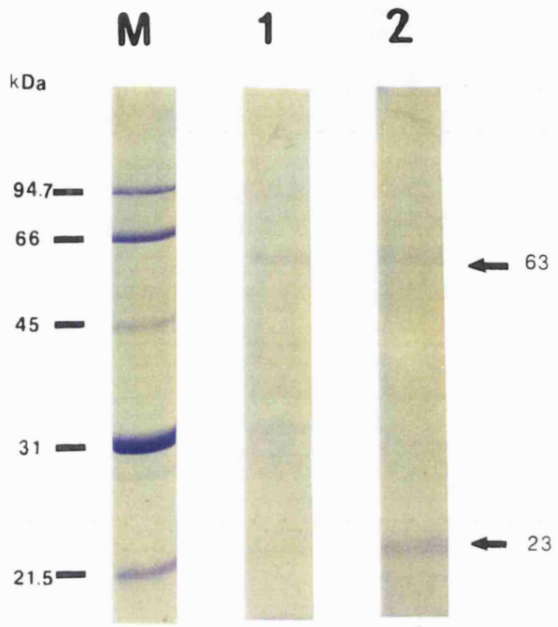


Fig. 4.11 SDS-PAGE pattern of partially purified VLPs preparation from *A. bisporus* virus associated proteins.

The gel is 10% acrylamide and stained with Coomassie Brilliant Blue.

The arrow indicates the putative mycovirus protein band.

Lane M : molecular weight standards (kDa).

Lane 1 : *A. bisporus* (control ; free from dsRNA)

Lane 2 : *A. bisporus* isolate V95

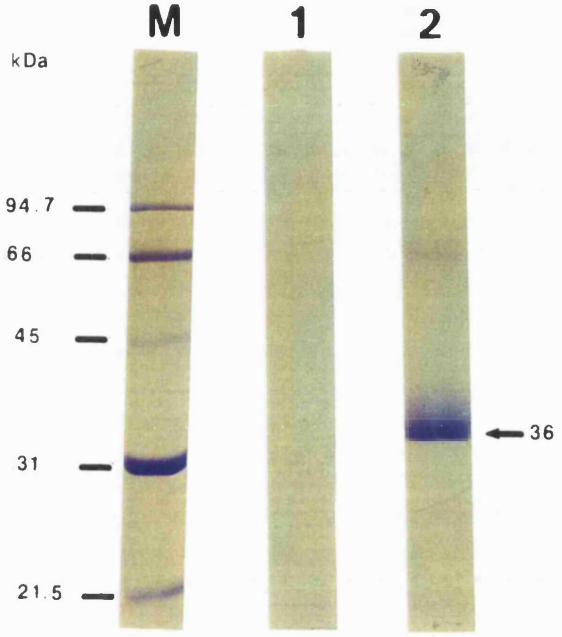


Fig. 4.12 SDS-PAGE pattern of partially purified VLPs preparation from *V. fungicola* virus associated proteins.
The gel is 10% acrylamide and stained with Coomassie Brilliant Blue.
The arrow indicates the putative mycovirus protein band.

Lane M : molecular weight standards (kDa).
Lane 1 : *V. fungicola* isolate V61-7 ; free from dsRNA)
Lane 2 : *V. fungicola* isolate V7-3

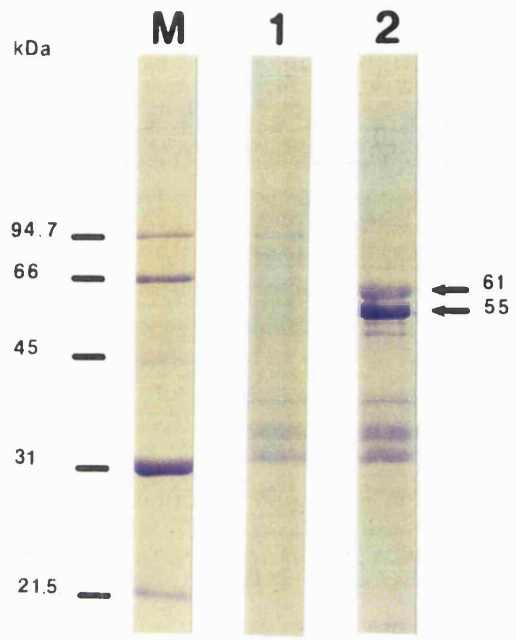


Fig. 4.13 SDS-PAGE pattern of partially purified VLPs preparation from *T. harzianum* virus associated proteins.

The gel is 10% acrylamide and stained with Coomassie Brilliant Blue.

The arrow indicates the putative mycovirus protein band.

Lane M : molecular weight standards (kDa).

Lane 1 : *T. harzianum* isolate T7 Reduced ; free from dsRNA)

Lane 2 : *T. harzianum* isolate T7

4.4 DISCUSSION

Procedures that have been used successfully for the purification of viruses from other fungi (Gillinds, *et al.*, 1993 and Howitt, *et al.*, 1995), were suitable for producing partially purified preparations from *A. bisporus* isolate V95 , *V. fungicola* isolate V7-3 and *T. harzianum* isolate T7; VLPs were seen in all preparations.

Saksena, (1975) reported that he combined and slightly modified various methods (Holling, 1962, and Van Zaayen and Timmink, 1968) and found that, whereas the mushroom *A. bisporus* virus particles require 6-10 % PEG for their precipitation, much extraneous material may be precipitated and removed initially by adding 1% (w/v) PEG-NaCl for 1 hour to the crude juice before initial centrifugation at 5,000 rpm for 20 min. Finally, preparations of high purity for 25 nm and 19 nm x 50 nm particles were obtained and showed ultraviolet absorption spectra typical of nucleo-protein with A_{260}/A_{280} ratios of 1.65 and 1.60, respectively. Saksena, (1975) described the procedures necessary for purification of mushroom virus particles as time-consuming and laborious and did not succeed in purification of the unstable 34 nm virus particles because they degrade rapidly.

Previous studies showed mycoviruses from *Aspergillus ochraceous* and *Penicillium stoloniferum* were isometric, and had diameters of 30-32 nm and 32-34 nm respectively. Both preparations had UV spectra which were typical for nucleoprotein preparations. The *A. ochraceous* preparation had a maximum absorbance at 259 nm and a minimum absorbance at 245 nm and the A_{260}/A_{280} ratio was 1.33. The *P. stoloniferum* preparation had a maximum absorbance at 261 nm and a minimum absorbance at 245 nm and the A_{260}/A_{280} ratio was 1.20 (Buck and Kampson, 1973 ; Kim and Bozarth, 1985).

Liang, *et al.*, (1990) showed that the spherical virus 23 nm diameter preparation from oyster mushroom, *Pleurotus sapidus,* had a UV absorption spectrum with a maximum at 257 nm and the minimum at 242 nm, showing a typical nucleoprotein character. In the case of the straw mushroom, *Volvariella volvacea* , the viruses detected were polyhedral particles of 35nm diameter and a few polyhedral particles of 45 nm diameter. Ultraviolet absorption spectrum of the virus preparation showed a maximum absorbance at 257 nm and a minimum absorbance at 230 nm and the A_{260}/A_{280} ratio was 1.96 (Chen, *et al.*, 1988).

Previous studies reported that a dsRNA genome was present in spherical to polyhedral viral particles such as the 25 and 34 nm particles from *A. bisporus* (Barton and Hollings, 1979 ; Sriskantha, *et al.*, 1986 ; Van der Lende, *et al.*, 1994). Moreover, Van der Lende, *et al.*, (1995) observed isometrical virus particles with diameters of 24 nm and 30 nm from mycelia of *P. ostreatus* var. *florida* that decreased growth rate and contained seven dsRNA segments.

Previous studies of *V. fungicola* reported three types of particle measuring, ca. 48, nm, 35 nm and 35x17 nm in diameter although electron micrographs of the reported particle types were not presented (La Pierre, *et al.*, 1973).

Howitt, *et al.*, (1995) reported that VLPs were observed by electron microscopy in partially purified virus preparations in dsRNA-containing isolates of the filamentous fungal *Botrytis cinerea.* Morphological types present included isometric particles of varying size classes (approx. 30, 35, 40, and 45 nm) and bacilliform particles (approx. 25 x 63 nm). As in the case of *Fusarium poae* , electron microscopic investigation of the purified dsRNA preparations revealed encapsidated VLPs. They were isometric, 30 nm (±2) in diameter, and such particles were absent in extracts of dsRNA-free isolates of the other *Fusarium* species tested (Fekete, *et al.*, 1995). Moreover, in some mycelial section of *Botrytis cinerea* Cvg25 showed the presence of isometric VLP of about 40 nm in diameter aggregated within membranous vesicles (Vilches and Castillo, 1997).

Albouy, (1972) observed in ultrathin sections of *A. bisporus* fruit bodies isometric particles 25 nm, 29 nm, 34 nm, 35nm, 50 nm and 60 to 65 nm in diameter as well as particles with helicoid symmetry of about 17 nm x 200 nm. The mushrooms examined were "showing symptom of total sterility". All isometric particle types observed by Albouy formed loose aggregates, often near a septum or dolipore, or were dispersed in the cytoplasm, sometimes possessing a dark centre and occasionally forming circular aggregates in vacuoles. Upon close study and accurate measurements of the observed particle types, one may arrive at the conclusion that Albouy observed mainly 34 nm virus particles and a few particles of a different morphology about 65 nm in diameter.

Previous work by Van Zaayen and Igesz (1969) studied ultrathin sections of *A. bisporus* diseased mycelium (which were very slowly growing) that showed aggregates of virus particles in the cytoplasm, often close to a nucleus or near a septum. Particles sometimes occurred in vacuoles, grouped irregularly or arranged into circular aggregates. Some particles had a dark centre. No VLPs were detected in healthy mycelium. The virus particles observed in mycelium were 34 nm in diameter (Van Zaayen, 1972 b).

Van Zaayan (1972 a) observed 34 nm virus particles in the septal pore apparatus seeming to pass from cell to cell in *A. bisporus*. The process was comparable with that of some plant viruses, which occurred in higher plants and pass through plasmodesmata (Esau, *et al.*, 1967; Dezoeten and Gaard, 1969). Observations on spherical virus particles in *P. ostreatus* give further evidence of virus translocation in basidiomycetes which most probably takes place through septal dolipores (Liang, *et al.*, 1990).

In ultrathin sections of spores from virus-diseased mushrooms, 34 nm particles were often found in numbers of up to several hundred per section from one spore (Van Zaayan, 1972 b). The particles usually were in clusters in small vacuoles and were sometimes arranged into circular aggregates , they sometimes occurred as well in the spore cytoplasm. Spores from healthy mushrooms did not contain virus like particles.

The only other difference between "infected" and "healthy" spores might be in the spore wall, as the wall of "healthy" spores appears perhaps to be slightly thicker than that of "diseased" spores (Schisler, *et al.*, 1967).

Ushiyama and Nakai (1982) reported that polyhedral virus-like particles, approximately 39 nm in diameter, from *L. endodes* have been studied *in vitro* and *in vivo* by electron microscopy. Examination of negatively-stained and rotary shadowed preparations of partially purified 39 nm VLPs showed that : (1) the particles have a double-shelled structure consisting of an outer capsid shell and an inner core containing dsRNA (Ushiyama, *et al.*, 1977), suggesting that this basic structure is closely similar to that of viruses belonging to the *Reoviridae*; and (2) some filamentous structures were bound to the exterior of capsids, and they were frequently found to be a feature of partial-core particles. Observations on ribonuclease-treated and rotary-shadowed preparations of 39 nm VLPs *in vitro* suggested that the extra-viral filaments attached to capsids might be dsRNA. Further observations on 39 nm VLPs which were isolated from glutaraldehyde-fixed mycelia, and on those in thin sections of hyphal cells, suggested that at least some particles might discharge dsRNA from their cores *in vivo.*

Many of the 39 nm VLPs had an electron-dense core with a diameter of approximately 33-35 nm, probably formed by nucleic acid; others had a partial core showing an electron-dense spot ranging in diameter from approximately 10-18 nm, while others appeared empty. These were mostly about 35-37. In addition, some disrupted or loose particles were observed either in aggregates or through the cytoplasm. Occasionally, some partial-core particles, appeared to lack part of the capsid shell and they contained external strands with an electron density (Ushiyama, *et al.*, 1977).

The discrepancies in measurements of the diameter of particles *in vitro* and *in vivo* are presumably due to an artifact resulting from various factors affecting particle size during different preparative procedures. Probably some partial-core particles

observed in negatively stained preparations and in thin sections might be intermediates in the pathway of dsRNA extrusion. The fixation with glutaraldehyde or osmium tetroxide effectively stabilizes virus capsids (Vernon, *et al.*, 1979), but does not fix the nucleic acid efficiently (Langenberg and Sharpee, 1978). Therefore, it seems possible that dsRNA strands which are located in the inner core are extruded. However, there is a question of how the dsRNA becomes detached from the inner core. Van der Lubbe, *et al.*, (1979) who observed that the particles of Fiji disease virus consisted of cores to which some dsRNA strands are attached externally, speculate that the RNA could possibly be extruded through holes in the B spikes when the A spikes are detached.

Several mechanisms, deduced largely from ultrastructural observations and from studies on the characteristics of in *vivo* replication of mycoviruses, have been proposed to explain the tolerance of fungi to virus infection. Ultrastructural studies on infected cells from several fungal species have frequently revealed that whereas the virus particles in younger cells were predominantly free in the cytoplasm, those in older cells were present in membrane-bound vesicles and in vacuoles, as well as free in the cytoplasm (Border, *et al.*, 1972 ; Hooper, *et al.*, 1972 and Yamashita, *et al.*, 1973). The enclosure of virus particles in vesicles and in vacuoles was suggested as a mechanism for controlling the overall level of virus in the fungal mycelium (Buck, 1977 ;and Lemka and Nash, 1974). Border (according to Buck, 1977) concluded from electron microscopic observation that only particles free in the cytoplasm could undergo multiplication, whereas those enclosed in vesicles might be effectively isolated from the rest of the cell.

Isometric particles about 20 nm diameter in *V. fungicola* V7-3 were visualized mainly as aggregated particles enclosed in membranous vesicles (Fig 4.6). This association of fungal viruses with membranous systems has been interpreted as an attempt by the host cell to delimit a potentially lytic virus in separate bodies (Weber, 1979). In most VLP-containing cells, some degree of cellular degeneration was observed in *T. harzianum* T7 (Fig. 4.10), a phenomenon that has also been observed in other species (Vilches and Castillo, 1997). The degeneracy with disappearance of

cellular organelles and cytoplasm has been suggested as a phenomenon of internal lysis and it is probable that the wide thickness and rigid cellular wall prevented cellular lysis (Weber, 1979).

Occasionally in some mycelium sections, thread-like structures that had the typical appearance of nucleic acid were observed. This appearance to be attached externally to many capsids similar to those reported by Ushiyama and Nakai (1982) from *L. edodes*. These structures might be RNA-protein complexes (Fig. 4.9 A, B), a phenomenon that had also been observed in herpes simplex virus type I (Griffith, *et al.*, 1999).

On the basis of particle morphology, Wood (1973) suggested that dsRNA viruses can be classified into three major groups ; (1) double capsid for the viruses belonging to the *Reoviridae*; (2) single capsid for fungal viruses; and (3) membrane - bound single capsid for bacteriophage ϕ6. The viruses of *P. chrysogenum* and *P. stoloniferum* PsV-S are composed of a single-layered structure (reviewed by Hollings, 1979). Also, the analysis by SDS-polyacrylamide gel electrophoresis indicated that the isometric viruses MV 1(25 nm diameter) and MV4 (35 nm diameter) of cultivated mushroom *A. bisporus* each contain one species of capsid polypeptide. Barton and Hollings, (1979) found that electrophoresis of the capsid polypeptides in 10 % SDS-PAGE showed that MV1 particles (25 nm VLP of *A. bisporus*) contained a distinct polypeptide of mol. wt 24.4 kDa and MV4 particles (35nm VLP of *A. bisporus*) contained one capsid polypeptide of mol. wt 63.8 kDa.

Goodin, *et al.*, (1992) reported that using a purification procedure involving chloroform extraction, PEG-NaCl precipitation, differential centrifugation, and equilibrium centrifugation in caesium-sulphate gradients, they have obtained preparations from diseased sporophores that were highly enriched in a 36 nm isometric VLP and contained minor amounts of both a 25 nm isometric VLP and the 19x50 nm ssRNA bacilliform virus. Caesium-sulphate gradient fractions that contained these particles also contained the nine disease specific dsRNAs of 3.8-0.8 kb and three disease-associated polypeptide with molecular weights of 63, 66 , 129 kDa and also a 61 kDa that was present in both healthy and diseases tissue of *A bisporus*.

Virions of La France disease (isometric 36 nm particles) are composed of three structural polypeptides which, depending on the method of purification, are estimated to be 63, 66 and 129 kDa (Goodin, *et al.*, 1992) or 90, 115 and 120 kDa (Van der Lende, 1994). However, Van der Lende, *et al.*, (1994) suggested that the proteins of lower MW seen by others could represent proteolytic artifacts. The pattern of degraded viral proteins closely resembles that reported by Goodin, *et al.*, (1992) and earlier by Barton and Hollings (1979). It is thus suggested that the morphologically intact virus particles isolated by these authors were proteolytically degraded. It was recently shown that proteolytic activity in *A. bisporus* fruit bodies is strongly induced after picking (Burton, *et al.*, 1993).

Van der Lende, *et al.*, (1994) assumed that the viral proteins of M_r 120 kDa, 115 kDa and 90 kDa are encoded by the disease-specific dsRNAs. The L1 dsRNA (3.6 kb) and possibly the L2 dsRNA (3.0 kb) may contain sufficient coding capacity to encode the 120 kDa and 115 kDa proteins, particularly since the apparent high M_r weight of these proteins on gels may be due to post-translational modifications, as was shown for mycovirus proteins found in *Helminthosporium victoriae* (Ghabrial, *et al.*, 1987; and Ghabrial and Havens, 1992). The protein of M_r 90 kDa could be encoded by any one of the L dsRNAs but M1, M2, S1 and S2 seem to lack sufficient coding capacity.

It is possible that the most abundant viral proteins detected in this study (M_r values of 120 kDa and 90 kDa) represent capsid proteins, whereas the less abundant and proteolytically resistant protein of M_r 115 kDa may be located inside the virus capsid and represent the RdRp. At this point, however, it is still possible that the three proteins represent the major capsid proteins of three different virus measuring 34 nm Van der Lende, *et al.*, (1994) .

Tavantzis and Bandy (1988) found that isometric virus particles 33 nm diameter were purified to apparent homogeneity from a dsRNA-containing isolate Rhs717 of basidiomycete *Rhizoctonia solania*. The capsid polypeptides of the Rhs717 virus showed a strong tendency to form stable aggregates and in this respect resembled coat proteins of mycoviruses T1-A and F6-B of *Gaeumannomyces graminis* (Buck, *et al.*, 1981). The virion dsRNA programmed the synthesis *in vitro* of two major polypeptides of approximate M_r 77K and 71K which, if each is the product of a different RNA species, would represent approximately the full coding capacity of the two viral dsRNAs. The smaller polypeptides observed varied in amount between experiments and may be premature translation products such as are often produced in cell-free translation systems (Wilson and Clover, 1983).

Previous work by Buck and Kempson-Jones (1974) reported the *P. stoloniferum* virus (PsV-S) slow capsid polypeptides component contained one major and one minor polypeptide with M_r 42 kDa and 55 kDa, while the *P. stoloniferum* virus (PsV-F) fast component contained one major and one minor polypeptide with M_r 47 kDa and 59 kDa, respectively. The ratios of major and minor polypeptides of PsV-S and PsV-F components were estimated to be about 100:1 and 40:1, respectively. In experiments by Kim and Bozarth (1985) it was found that the PsV-S and PsV-F components consisted mostly of the higher molecular weight polypeptide, i.e. a reverse ratio of the major and the minor polypeptides. It is possible that the smaller polypeptides of Buck and Kempson-Jones might have been derived from the larger polypeptides by proteolytic degradation during the purification of mycoviruses, as in the cases of *A. foetidus* virus (AfV-S) (Buck and Ratti, 1975) and cowpea mosaic virus (CPMV) (Geelen, *et al.*, 1973). AfV-S contained one major and one minor capsid polypeptide with M_r of 83 kDa and 78 kDa, respectively. The proportion of the smaller M_r 78 kDa polypeptide frequently increased with the age of the virus preparations. CPMV had fast and slow moving electrophoretic forms. They were equally infectious. During storage of CPMV, a conversion of slow into fast form was found; this partial conversion probably results from proteolytic enzymes (Kim and Bozarth, 1985).

Zelikovitch, *et al.*, (1990) reported the *Septoria tritici* isolates ISR398 and ETH8205 contained one dsRNA segment (1.3 kb). The isometric 35 nm virus particles were purified and viral capsid consisted of one major protein with a molecular weight 60 kDa. Finkler, *et al.*, (1985) showed a coat protein of 55 kDa detected from virions of *Rhizoctonia solani*. The virus were 30-35 nm in diameter and contained two segments of dsRNA with molecular weight 1.45 and 1.32 kb.

Typically, the genomes of mycoviruses consist of one to three dsRNA segments that are packaged separately in isometric particles composed of a single capsid polypeptide (Buck, 1986). The capsid polypeptide of the *V. fungicola* V7-3 showed a single polypeptide with M_r 36 kDa was obtained from the single virus. However, the genome of V7-3 virus described here consists of five dsRNA segment, which some are encapsidated and some might be satellite dsRNA. It is possible that the two most abundant viral polypeptide detected in *T. harzianum* T7 represent capsid protein of two viruses. It can not be formally ruled out that one smaller polypeptide are breakdown products of the larger polypeptides.

CHAPTER 5

Reverse Transcriptase and Polymerase Chain Reactions (RT-PCR), Cloning and Sequencing

CHAPTER 5 Reverse Transcriptase and Polymerase Chain Reactions (RT-PCR), Cloning and Sequencing

5.1 INTRODUCTION

cDNA cloning by means of Reverse Transcription Polymerase Chain Reaction (RT-PCR) is a widely used technique. Ever since the first published method by Veres, *et al.*, (1987) it has supplemented conventional methods of cDNA cloning due to its ease and speed. However, there are certain constraints that are associated with this technique; e.g., it requires the knowledge of a few nucleic acid sequences of the gene of interest. These can be obtained by either microsequencing the protein itself followed by the construction of a putative cDNA sequence or, more commonly, by using the homologous cDNA sequence from other species. However, both methods use degenerate primers (Girgis, *et al.*, 1988 and Lee, *et al.*, 1988), which can give rise to nonspecific priming (Carmody and Vary, 1994). Therefore, variations of PCR have been developed to overcome this problem, e.g., touchdown PCR (Don, *et al.*, 1991) and nested PCR (Albert and Fenyo, 1990).

However, PCR products have proven unusually resistant to standard cloning procedures because of two main reasons: first, blunt-end cloning of unmodified PCR products is very inefficient, due to the template-independent terminal transferase activity of *Taq* polymerase, which results in the addition of a single nucleotide (almost exclusively dATP) to the 3′ends of PCR fragments (Clark, 1988 and Mole, *et al.*, 1989). Second, restriction sites, artificially incorporated into the oligonucleotide primers for subsequent digestion and sticky-end cloning, often proving difficult to cut and sometimes generating nonspecific PCR products.

The development of the T-vector technique (Marchuk, *et al.*, 1991) led to a rapid and reliable strategy for efficient cloning of PCR products. A cloning vector is digested within its multiple cloning site by a blunt-end cutter and a single thymidine is subsequently added to the 3′ends of the linearized vector by *Taq* polymerase. PCR product and vector consequently exhibit complementary single base 3′ overhangs and therefore can be ligated efficiently (Papp, *et al.*, 1995).

A previous study by Leister and Thompson (1996) reported the production of full-length viral cDNA of isolate UKG27/72 of swine vesicular disease virus (SVDV) using RT-PCR. It was concluded that picornaviral genomes consist of ssRNA of positive polarity longer than 7 kb, and contained an unknown concentration of total RNA in infected cells. The advantages of this method are the rapidity by which results are obtained and that they were achieved without using radiolabelled compounds or methods of molecular cloning.

A variety of double-stranded RNA genomes have been described. They have been the subject of many biochemical and virological studies, but the little nucleotide sequence information available on this important group of genetic elements is limited to terminal regions. This is because restriction endonucleases that cut dsRNA have not been found, and in contrast to most eukaryotic mRNAs, transcripts of dsRNA contain no 3′-terminal poly (A), which would facilitate purification and provide a basis for oligo(dT)-primed synthesis of cDNA for extensive sequence determination (Imai, *et al.*, 1983)

In recent years, the complete nucleotide sequence of a member of the genus *Totivirus* in the family *Totiviridae* has been determined. The *Saccharomyces cerevisiae* L-A (ScV-LA) virus is the only member of the genus that has been well-characterized at the molecular level (Ghabrial, *et al.*, 1995 a). The complete sequence of the ScV-La virus (synonymous ScV-L-BC virus), and partial sequence of the *Ustilago maydis* virus H1 have been reported (Bruenn, 1993 and Park, *et al.*, 1996 a). The complete nucleotide sequence of several of the totiviruses that infect the parasitic protozoa *Giardia lamblia* (Giardia lambia virus, GLV) and *Leishmania*

species [two strains of Leishmania RNA virus 1 (LRV1-1 and LRV1-4) and LRV2-1] have been determined (Scheffter, *et al.*, 1994 ; Scheffter, *et al.*, 1995 ; Stuart, *et al.*, 1992, and Wang, *et al.*, 1993).

The complete nucleotide sequence, 5178 bp, of the totivirus in the plant-pathogenic ascomycete *Helminthosporium victoriae* 190S virus (Hv 190SV) dsRNA, has been determined (Huang and Ghabrial, 1996). The full sequence of 6.4 kb, dsRNA (M1) in the plant-pathogenic basidiomycete *Rhizoctonia solani* was determined by sequencing the cDNA clones (Jian, *et al.*, 1998). The nucleotide sequences of five of the dsRNAs in the mushroom basidiomycete *Agaricus bisporus* indicated that L1 (3.6 kb) encoded the 115 kD virion-associated RNA dependent RNA polymerase (RdRp), L3 (2.8 kb) encoded the 90 kD capsid polyprotein, and L5 (2.5 kb), M1 (1 kb) and M2 (1.3 kb) each encoded single proteins of unknown function (Harmsen, *et al.*, 1991 and Van der Lende, *et al.*, 1996).

This chapter focuses on the molecular characterization to understand the nature and role of dsRNA in *A. bisporus* isolate V95, *Verticillium fungicola* isolate V7.3 and *Trichoderma harzianum* isolate T7. The cDNA clones of the associated dsRNAs were constructed for sequencing. The nucleotide sequences were determined and compared with those of some other positive-strand ssRNA virus and dsRNA mycoviruses.

5.2 MATERIALS AND METHODS

5.2.1 cDNA synthesis and primers selection of dsRNA of *A. bisporus*

Primers specific for L1 , L5 were designed on the basis of published sequence data for L1 3.6 kb and L5 2.5 kb dsRNA of *A. bisporus* (Van den Lende, *et al.*, 1996).

Specific primer used for RT-PCR and sequencing (dsRNA of *A. bisporus*).

L1 Sense-strand primer (5'------3')

Primers	Positions	Sequence (5' to 3')	length
L1f0	1-22	ACTTGTTTGAATATGTTGAGTG	22
L1f1	101-118	CACGTGCGAAAGCAGTGG	18
L1f3	902-921	CTGCATGTTTGATTGGTTCG	20
L1f4	1601-1620	CTGCCTGGCGGAATTATGA	20
L1f5	2302-2322	ATAACTCCAGAAGGTGTATTC	21
L1f6	2901-2921	ATCGCCTTTAACGATAGATGA	21

L1 Complementary-sense primers (5'------3')

Primers	Positions	Sequence (5' to 3')	length
L1r2	600-583	CCGGCGACCAAAGCTGTA	18
L1r1	1100-1081	CTTATCCTCGCATGAGCATA	20
L1r3	1799-1779	TCCTTCAATTGCCTATCACTA	21
L1r4	2499-2478	GCTCATATAATGTTGCAATTGT	22
L1r5	3280-3200	CTAGCATTCAGTTTTTCTGCA	21
L1r6	3396-3377	GCCATTTAAGTTGCACACTA	20

L5 Sense-strand primers (5′------3′)

Primers	Positions	Sequence (5′ to 3′)	length
L5f0	1-20	GGATAAGTGGTTAGTGCAAG	20
L5f1	201-218	TGCCTGGGAGTCTGTTGC	18
L5f2	686-705	AAACAACTACAGGAGCTTGC	20
L5f4	802-819	CAATGCGAACGGACCCAC	18
L5f3	1701-1721	ACATGCAATGCAAGACAAATG	21

L5 Complementary-sense primers (5′------3′)

Primers	Positions	Sequence (5′ to 3′)	length
L5r2	481-462	CGACTTGCTGCTATTTTCTG	20
L5r1	900-880	ATCGCTTCTGTTGGTAAACTA	21
L5r4	1797-1778	AACACTTCGCTGATTGCTTC	20
L5r3	2220-2201	TTAAACCCAATCGCACTTGC	20
L5r5	2455-2433	TTTGGATTTAATTTAGCTCTCTA	23

5.2.2 cDNA synthesis and primers selection of dsRNA of *V. fungicola* and *T. harzianum*

Ten random oligonucleotides primers were designed but only two were suitable.

Random oligonucleotides primers (5′------3′)

:used for RT-PCR(dsRNA of *V. fungicola, T. harzianum*)

Ra#3	TTCCTCAGCCTTGAGTGCCT	20 mer
Ra#4	TCATGGAACTGCTTGGAACC	20 mer

Specific primers (5′------3′)

:used for RT-PCR (dsRNA of *V. fungicola*).

Vf2	GGCTGAGTGAGGCACTCAAGG	21 mer
Vr2	CCCGTCACTATTGGTGACTTCG	21 mer

Specific primers (5′------3′)

:used for RT-PCR (dsRNA of *T. harzianum*).

Tf1	TTATTCCAACATCTTCTGAGCC	22 mer
Tf3	GTGTTTACCTTTTGGGGGGTAAAT	24 mer
Tr2	GGGCTGTTAACACTTTAGAAGCCG	24 mer

Sequencing Specific primer for cDNA clones (5′------3′)

T7	GTAAAACGACGGCCAGT	forward
Sp6	CAGGAAACAGCTATGAC	reverse

5.2.3 RT- PCR amplification

For the reverse transcription and amplification, 3µl of each primer (50 pmol) and 1 µl of total dsRNA sample (300-500 ng) were added to a 0.5 ml microreaction tube. The mixture was boiled for 10 min to denature the dsRNA and then allowed to cool on ice for 10 min. The mixture was brought to 43 µl ; 30 µl of nuclease-free water, 10 µl of 5x AMV/*Tfl* reaction buffer, 1 µl of 10 mM each dNTP mix , 2 µl of 25 mM $MgSO_4$, 5 unit of AMV Reverse transcriptase, 5 unit of *Tfl* DNA Polymerase (Access RT-PCR system kit, Promega). The reaction mixture was overlaid with 50 µl mineral oil to prevent evaporation. The reactions were carried out in a PTC-100 Thermal Cycler (MJ Research Inc.) in the following way : reverse transcription for 45 min at 48 °C , 2 min 94 °C (hot start) and amplification of DNA 40 cycles of 30 sec at 94°C (denaturation), 2 min at 62°C (annealing) and 3 min at 72 °C (extension). After the final polymerisation step, the reaction was kept for 7 min at 72 °C to complete the extension of all strands formed , and then cooled to 4 °C until analysis.

5.2.4 Gel electrophoresis

Following amplification , 10 µl of the reaction mixture was added to 3 µl of a loading buffer. This was analysed on 1 % agarose gel in TBE buffer pH 8.3 and EtBr 0.5 µg/ml was added. The gel was electrophoresed at 60 volts for 1.5 hours. DNA was visualised by transillumination with UV light and photographed (Life sciences ; UVP Ltd., UK) and a 100 bp DNA ladder marker was used as a size standard.

5.2.5 DNA purification from agarose gels

Amplified DNA was electrophoretically purified from agarose gels using Sephaglas Band prep Kit (Pharmacia Biotech). The appropriate DNA band was cut out placed in a microcentrifuge tube and the agarose weighed. To the tube was added Gel Solubilizer (1 µl/mg of gel) and 1 µl of 50 % glacial acetic acid / 50 µl Gel Solubilizer. This mixture was vortexed vigorously and incubated at 60 °C for 15-20 min. Vortexed Sephaglas BP was added (5 µl for each 1 µg of DNA) and vortexed gently. The mixture was incubated at room temperature for 5-10 min and vortexed gently each min , then pulse spun for 30 sec and the supernatant discarded. The pellet was washed with buffer three times and air dried for about 30-60 min. The pellet was mixed with elution buffer and resuspended by vortexing at room temperature for 5 min. The tube was centrifuged , the supernatant transferred to a new tube and stored at -20 °C for further processing.

Purified cDNA from dsRNA of *A. bisporus* was sequenced directly. The cDNA from dsRNA of *V. fungicola, T. harzianum* were amplified by second round of PCR using the same primers.

5.2.6 Second round PCR amplification

Gel purified cDNAs from dsRNAs of *V. fungicola* and *T. harzianum* were amplified by a second round of PCR using the same primers. The PCR reaction contained 1 µl template cDNA, 20 pmol of primer, 1.5 µl of 10 mM dNTP, 2 µl of 50 mM $MgSO_4$, 5 µl of 10x *Taq* reaction NH_4 buffer , 5 U *Taq* DNA polymerase (Bioline). The reaction mixture was brought to 50 µl / reaction with nuclease-free water and overlaid with 50 µl mineral oil to prevent evaporation.

Reactions were carried out in a PTC-100 Thermal Cycler (MJ Research Inc.) in the following way : 3 min 94 °C (hot start) and amplification of DNA 40 cycles of 30 sec at 94°C (denaturation), 2 min at 62°C (annealing) and 3 min at 68 °C (extension). After the final polymerisation step, the reaction was kept for 7 min at 68°C to complete the extension of all strands formed , and then cooled to 4 °C until analysis. PCR products were examined by 1% agarose electrophoresis. Following DNA purification from agarose gel was performed.

5.2.7 Ligation of PCR product into the pGEM-T Easy vectors

Second round PCR products of dsRNA of *V. fungicola* and *T. harzianum* were cloned into the T-tailed vector pGEM-T Easy (Promega). Ligations were set up with the following ratios of insert DNA to plasmid (2 :1 ; 100 ng :50 ng) ,with final concentration of 30 mM Tris-HCl (pH 7.8) , 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 0.3 Weiss units of DNA ligase made up to a 10 µl reaction volume with deionized water. These were made up in 0.5 ml Eppendorf tubes and incubated at 4°C overnight.

5.2.8 Transformation of competent *E. coli* cells with pGEM -T Easy

Competent *E. coli* cells (strain DH5α) were prepared using calcium chloride according to the protocol of Sambrook *et al*, (1989). Briefly, a fresh overnight DH5α was diluted 1:50 into 50 ml of LB broth (10 g bacto tryptone, 5 g bacto yeast extract, 10 g NaCl per litre) in a 250 ml conical flask, and incubated at 37°C with shaking at 225 rpm, to a bacterial density (A600) of 0.5-0.6 (~$2.5x10^8$ cell/ml, approximately 3 hr growth). The culture of DH5α were transferred into cold 50 ml polypropylene tubes and the culture cooled on ice for 10 min, and pelleted at 2,000 g for 10 min at 4°C. The supernatant was discarded, and the cells resuspended in 10 ml of ice-cold 0.1 M Ca_2Cl, and pelleted at 2,000 g for 10 min at 4°C. The supernatant was discarded, and the cell resuspended in 2 ml of ice-cold 0.1 M Ca_2Cl for each 50 ml of original culture.

Transformation of the competent cells with pGEM-T Easy followed the protocol in Sambrook *et al* (1989). 200 µl of competent cells were transferred to a 1.5 ml Eppendorf tube and 5µl of ligation reaction (50 ng of DNA) was added to the competent cells and mixed by gently swirling with the pipette tip. The samples were incubated on ice for 30-60 min, and then heat shocked at 42°C for 90 sec, in the heating block. The shocked cells were cooled on ice for 2 min, and 0.8 ml SOC medium added to the Eppendorf tubes, before incubating at 37°C for 45-60 min with shaking at 150 rpm.

Transformants were selected on plates that contained 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; 1mg/plate), IPTG (10 µM/ plate) and ampicillin (50 mg/ml). Transformation cultures were spread as 200 and 500 µl per plate, before incubating for 12-16 hr at 37°C. Recombinant plasmids were prepared from the selected transformants by the alkaline lysis method (Sambrook, *et al.*, 1989) and digested with *Eco* RI (Promega). The sizes of digested cDNA fragments were analyzed by agarose gel electrophoresis. Northern hybridization experiments were carried out using independent clones.

5.2.9 Blotting and Northern hybridization

The dsRNAs (*V. fungicola* and *T. harzianum*) were separated in a 0.8 % agarose gels that contained TBE buffer pH 8.3 and EtBr 0.5 μg/ml. The gel was then immersed in 200 ml of 50 mM NaOH and agitated gently for 30 min at room temperature. The dsRNA were then transferred to Hybond-N nylon membrane (Amersham) by the capillary method with 50 mM NaOH for 18 hr and baked for 2 hr at 80 °C under vacuum.

The membranes were prehybridised with gentle shaking at 50°C for 4-6 hr with buffer containing ; 5xSSPE (1x SSPE : 180 mM NaCl, 10 mM $NaH_2PO_4.H_2O$, 1mM EDTA pH 7.4), 50 % formamide, 0.2% SDS, 100 μl/ml salmon sperm (ssDNA), 5x Denhardt's solution.

Probes for hybridization were synthesized from the cDNA fragments of recombinant plasmids by use of a Oligolabelling Kit (Pharmacia Biotech) with [α-^{32}P] dCTP. The probe was added to prehybridization buffer ,after denaturing for 5 min at 95-100 °C , and carried out overnight at 50°C with shaking at 80 rpm.

Membranes were washed twice with 200ml of 2xSSC at 50°C for 10 and 15 min, followed with 200ml of 1xSSC at 50°C for 1 hr and 0.5 xSSC at 50°C for 2 hr. The membranes were then blotted dry, wrapped in cling film, and placed in autoradiography cassettes. The membranes were exposed for 1 day to X-ray film (Kodak) with intensifying screens at -70°C prior to film development on an automated developer. Membranes could be reprobed two times , if stripped of their radioactive signal by boiling in 1% SDS and then rinsing in 0.1xSSC.

5.2.10 DNA sequencing

Automated sequencing was carried out using the Sanger-Coulson method (Mathews and van Holde, 1990) and the sequencing primer was derivatised at its 5'end with fluorescent dRhodamine dye terminators. Each reaction contained 30-90 ng DNA (RT-PCR: from dsRNA of *A. bisporus*) or 200-500 ng DNA (cDNA-plasmid: from dsRNA of *V. fungicola, T. harzianum*) and 1.6 pmol primer, in total of 6 µl.

Specific fragments of DNAs were sequenced on an ABI model 377 DNA sequencer (Perkin Elmer) using the PRISM Ready Reaction Dye Terminator Cycle Sequencing kit . All fragments were completely sequenced on both strands.

5.2.11 Software for DNA sequence analysis

Sequence data were manipulated in the Wisconsin GCG Package, (Program Manual for the Wisconsin Package, Version 8.0-UNIX, August 1994, Genetics Computer Group, 575 Science Drive, Madison, USA 53711 ; Web Site: http://www.ncbi.nih.gov/). Database searches were conducted with GCG software on the EMBL and GenBank databases (DNA and RNA) and DDBT , PDB (protein) , using the Blast Sequence Similarity Searching method (Altschul *et al*, 1997) and the programmes Translate, SIM+LANVIEW, CLUSTALW, and WUBLAST listed on the ExPASY home page (http://www.expasy.ch/www/). Sequence data were displayed using GeneDoc: Multiple Sequence Alignment Editor & Shading Utility, Version 2.4.000 (Nicholas and Nicholas, 1997; Web Site: http://www.cris.com/~ ketchup/genedoc.shtml).

5.3 RESULTS

5.3.1 RT-PCR of dsRNA of *A. bisporus*

RT-PCR amplifications were carried out with six pairs of primers (L1f0/L1r2, L1f1/L1r1, L1f3/L1r3, L1f4/L1r4, L1f5/L1r5, L1f6/L1r6) designed from the sequence of the L1 3.6 kb dsRNA 1 and six pairs of primers (L5f0/L5r2, L5f1/L5r1, L5f2/L5r4, L5f4/L5r4, L5f4/L5r3, L5f3/L5r5) designed from the sequence of the L5 2.5 kb dsRNA 5 of *A. bisporus* of The Netherlands isolate (Van den Lende *et al*, 1996). The expected fragments were successfully amplified from total dsRNA of *A. bisporus* of UK isolate and yielded a single major DNA product for each pair primer. Fig 5.1 showed that the amplicons had estimated sizes of 1000 bp (L1f1/L1r1) and 700 bp (L5f1/L5r1) of dsRNA of *A. bisporus* isolate V95 from The UK. The consistency of these results (Fig 5.2) suggests that the sequences of UK isolate are similar to The Netherlands isolate. For L1 (≈ 3600 bp) 3391 bp were sequenced and L5 (≈ 2450 bp) 2371 bp were sequenced. L1 dsRNA sequence analysis showed that dsRNA had a long open reading frame (ORF) of 1082 amino acids starting at nucleotide 13 with the codon AUG and ending at nucleotide 3261 with the code UAA (Fig 5.3). The L5 dsRNA had a large open reading frame of 724 amino acids starting at nucleotide 127 with the codon AUG and ending at nucleotide 2301 with the code UGA (Fig 5.4).

The assembled sequences were compared with the other with the BLAST Sequence Similarity Searching method indicated that the nucleotide sequence of L1 and L5 dsRNA of *A. bisporus* isolated from UK shared 95% and 98% homology , moreover, comparison of predicted amino acid sequences showed 93% and 97% homology with the published sequence data for the L1 and L5 dsRNA of *A. bisporus* virus 1 isolate from The Netherlands by Van der Lende *et al*, 1996 (appendix III-1, III-2, III-3 and III-4).

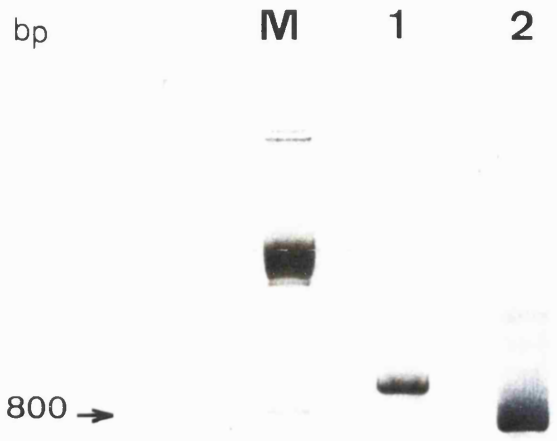
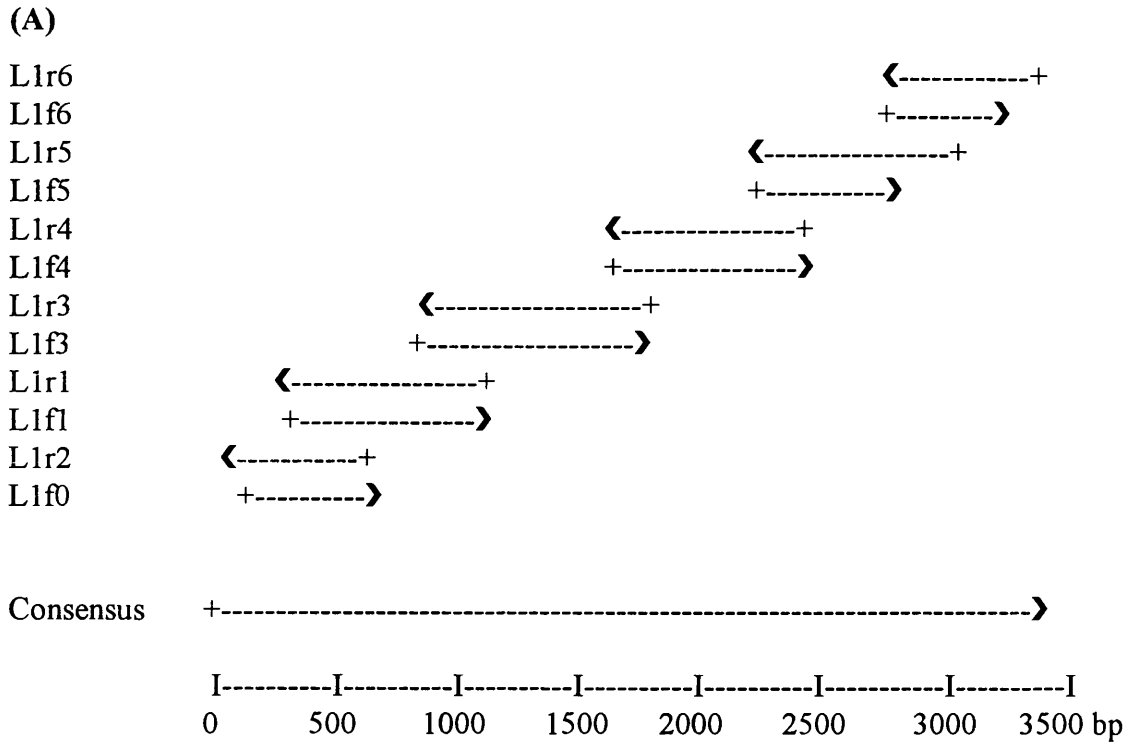


Fig. 5.1 Specificity of the RT-PCR targeting the viral genomic RNA of *A. bisporus*. Shown are the DNA amplification products with expected size.

M) Molecular weight marker (λ-DNA 100-bp ladder).

1) Amplification products with expected size 1000 bp for L1 (L1f1-L1r1).

2) Amplification products with expected size 700 bp for L5 (L5f1-L5r1).



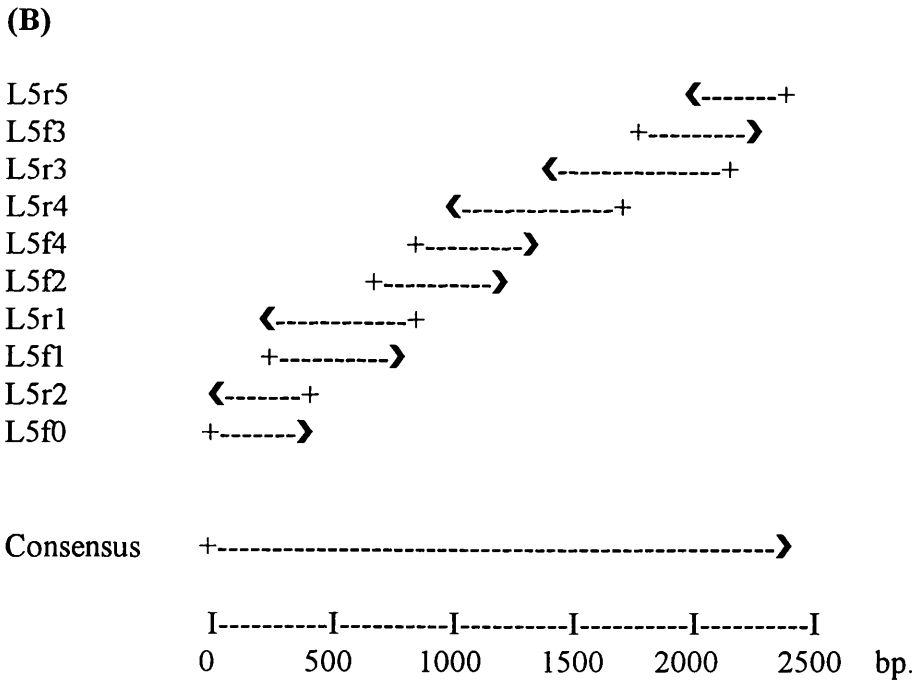


Fig. 5.2 Schematic presentation of cDNA derived using RT-PCR . The position of clones are drawn with respect to the complete dsRNAs.

A) L1 dsRNA of *A. bisporus* (V95) isolate from UK.

B) L5 dsRNA of *A. bisporus* (V95) isolate from UK.

```
   1  acttgtttgaatatgttgagtggatatgatagagaccagataattgggtgggctgaggat
     -  T  C  L  N  M  L  S  G  Y  D  R  D  Q  I  I  G  W  A  E  D
  61  attccaaattttgatgttgagtacgcttgcatttcacacacacgtgcgaaagcagtggtt
     -  I  P  N  F  D  V  E  Y  A  C  I  S  H  T  R  A  K  A  V  V
 121  ggtgaggatattgctcataatttgtcctgctattcaaggttacgtaccgcattgtctaat
     -  G  E  D  I  A  H  N  L  S  C  Y  S  R  L  R  T  A  L  S  N
 181  ataaatccagacgataatgcgcctttcatttttgtccagacttgtgaactggcagaagca
     -  I  N  P  D  D  N  A  P  F  I  F  V  Q  T  C  E  L  A  E  A
 241  attggagtacagattgcgatggtgttgatagtcaaggaggggataattcaacaaactgat
     -  I  G  V  Q  I  A  M  V  L  I  V  K  E  G  I  I  Q  Q  T  D
 301  aggtataaaaatttgtcaagagaagaacagaaaactcttaagcatgtgcatgatgttcaa
     -  R  Y  K  N  L  S  R  E  E  Q  K  T  L  K  H  V  H  D  V  Q
 361  aataatcataataaattgtgggttgaaaaatataagtatacgcatagggtcgtcttatct
     -  N  N  H  N  K  L  W  V  E  K  Y  K  Y  T  H  R  V  V  L  S
 421  tataatgatatttaccacaaggttaagggattatatattagtgttgggtgtgtgaaaagc
     -  Y  N  D  I  Y  H  K  V  K  G  L  Y  I  S  V  G  C  V  K  S
 481  tcctttggcacagatgatgtgttgagtgggatagatgagtatacttacgatgctcgcgag
     -  S  F  G  T  D  D  V  L  S  G  I  D  E  Y  T  Y  D  A  R  E
 541  aaggtgaatgaggtttcgaatttgcttaaagagtcgaagcatccggcgaccaaagctgta
     -  K  V  N  E  V  S  N  L  L  K  E  S  K  H  P  A  T  K  A  V
 601  tgcgcgaggtatttgcagcaaaattttcaggcggcagcgccagattatgctaatgcaagt
     -  C  A  R  Y  L  Q  Q  N  F  Q  A  A  A  P  D  Y  A  N  A  S
 661  tgttcccacgttgcgtggtcgagatgggtaaatgcagtcaaatcgcacgttagaactgaa
     -  C  S  H  V  A  W  S  R  W  V  N  A  V  K  S  H  V  R  T  E
 721  agtatgcattatccaacaaacaatatggtaatggccatgtgcgacgaagagtatgtgata
     -  S  M  H  Y  P  T  N  N  M  V  M  A  M  C  D  E  E  Y  V  I
 781  gagaactttcctcatcaagcagggtcagacaagtttgctgcctgtcatataagtacagtg
     -  E  N  F  P  H  Q  A  G  S  D  K  F  A  A  C  H  I  S  T  V
 841  atcaaggatcacatggataagttgaaaaaaccaacaactggcagacctgtgtttgttcag
     -  I  K  D  H  M  D  K  L  K  K  P  T  T  G  R  P  V  F  V  Q
 901  actgcgtgtttgattggttcggtcttgaagaacaaagatatccagtgttcgtatgagaag
     -  T  A  C  L  I  G  S  V  L  K  N  K  D  I  Q  C  S  Y  E  K
 961  ctattgtcttttatattatatgaaatgttcttgtacacaacggatgttgagatgcataaa
     -  L  L  S  F  I  L  Y  E  M  F  L  Y  T  T  D  V  E  M  H  K
1021  ttgtggagcagtgtggtgtattcgcctgtagataaagatactttctttttgtgtgcagag
     -  L  W  S  S  V  V  Y  S  P  V  D  K  D  T  F  F  L  C  A  E
1081  tatgctcatgcgaggataaggacatcgggaacgtatttaggtataaaattgactgcgcaa
     -  Y  A  H  A  R  I  R  T  S  G  T  Y  L  G  I  K  L  T  A  Q
1141  cagatgtcatatatgttgtatccgcaatgtttagctggtagggtgtatggcaaagaggat
     -  Q  M  S  Y  M  L  Y  P  Q  C  L  A  G  R  V  Y  G  K  E  D
1201  ttggcacaagagtttgacgatcgtacttctgataggccggcgaagaggttctggtccaat
     -  L  A  Q  E  F  D  D  R  T  S  D  R  P  A  K  R  F  W  S  N
1261  ggagtgctgagcgaagaagaatataacataaggtttaataatgcaatactgaactgttat
     -  G  V  L  S  E  E  E  Y  N  I  R  F  N  N  A  I  L  N  C  Y
1321  tcgcatatgacaaccggatatagtgctaaatttaggcggttggcgaatgatgttgaatcg
     -  S  H  M  T  T  G  Y  S  A  K  F  R  R  L  A  N  D  V  E  S
1381  ttcaaatcgtttatgcagttacgaaaacgctggtcacagccgggttcggcaactggtgca
     -  F  K  S  F  M  Q  L  R  K  R  W  S  Q  P  G  S  A  T  G  A
1441  ccgaaagttattcttagattgacggaacatgtggctgagttagatgccgatgcgcttcag
     -  P  K  V  I  L  R  L  T  E  H  V  A  E  L  D  A  D  A  L  Q
1501  ctgggtgagttgttgactgatgccatcgataggattcaattgagacttaacaagtcaaca
     -  L  G  E  L  L  T  D  A  I  D  R  I  Q  L  R  L  N  K  S  T
1561  ttatttgaattttctgccgtagtagaagcggtggaggctgcctggcggaactatgatccg
     -  L  F  E  F  S  A  V  V  E  A  V  E  A  A  W  R  N  Y  D  P
1621  aatagttttactggtgtgttctggaaacatgaagtgggaaaaaacgcatctcggtcactg
     -  N  S  F  T  G  V  F  W  K  H  E  V  G  K  N  A  S  R  S  L
1681  tggccagcgcatctagtccattatgttctagtgtccatgatattacacttaatcgacaag
     -  W  P  A  H  L  V  H  Y  V  L  V  S  M  I  L  H  L  I  D  K
1741  tcaggggaaataccaggatcacgtaacaatgcacctagtgataggcaattgaaagatcac
     -  S  G  E  I  P  G  S  R  N  N  A  P  S  D  R  Q  L  K  D  H
1801  tggatgtggtcagaatgccaagattttgtacctttaatgatggattacgctaatttaac
     -  W  M  W  S  E  C  Q  D  F  V  P  L  M  M  D  Y  A  N  F  N
```

```
1861  gaacagcatagcatagaggcgatgaaagcaacaatacagccactgcgcgatgtatatgct
    -  E  Q  H  S  I  E  A  M  K  A  T  I  Q  P  L  R  D  V  Y  A
1921  aaggcgggagtgctgtctaaagatctagctgatgctattgattgggtagtcaaatcgttt
    -  K  A  G  V  L  S  K  D  L  A  D  A  I  D  W  V  V  K  S  F
1981  gatcaaatatgcgctatagatgaaaatggtgacttgaggcgatttacacacggcttgtta
    -  D  Q  I  C  A  I  D  E  N  G  D  L  R  R  F  T  H  G  L  L
2041  tctggatggagatgtacggcttacatcaataatttaataaatatagctcagtacgaagtg
    -  S  G  W  R  C  T  A  Y  I  N  N  L  I  N  I  A  Q  Y  E  V
2101  ggtagacaacagctgcatgagtttttggaatgagttcttcataccagtttgacacaggg
    -  G  R  Q  Q  L  H  E  F  F  G  M  S  S  S  Y  Q  F  D  T  G
2161  ggagacgatggatgtgcagatgaaaaagacttggtgacagcttattcgttgttgcgtagc
    -  G  D  D  G  C  A  D  E  K  D  L  V  T  A  Y  S  L  L  R  S
2221  atgactgctatgggatttgagtttaaggatataaaacaattaataagtagcggaacgcac
    -  M  T  A  M  G  F  E  F  K  D  I  K  Q  L  I  S  S  G  T  H
2281  gaatttttcagattgctaataactccagaaggtgtattcggttcagtgatcaggatgtta
    -  E  F  F  R  L  L  I  T  P  E  G  V  F  G  S  V  I  R  M  L
2341  gcatctgctgcatcaggccaatggtctaattcagtgagagcaaaattagtggacccactt
    -  A  S  A  A  S  G  Q  W  S  N  S  V  R  A  K  L  V  D  P  L
2401  tcaaagatgactagtataatggatataaaacataaattatggaggagatcaggttataat
    -  S  K  M  T  S  I  M  D  I  K  H  K  L  W  R  R  S  G  Y  N
2461  gatgattgggctgaacaattgcaacattatatgagcttgaaatgggtagatccggagagc
    -  D  D  W  A  E  Q  L  Q  H  Y  M  S  L  K  W  V  D  P  E  S
2521  ccaaaagatttgataaattttgtgcatggcacgaaaagtacaggaggtttgggtatacca
    -  P  K  D  L  I  N  F  V  H  G  T  K  S  T  G  G  L  G  I  P
2581  gactgtcaaggtaggttatatgagttggcaagtgtgagtggaatagaaaaaccatcaaaa
    -  D  C  Q  G  R  L  Y  E  L  A  S  V  S  G  I  E  K  P  S  K
2641  atagaactgcgttcattcccgcacgatgcgagtgagcagttgctgaataaattagctcca
    -  I  E  L  R  S  F  P  H  D  A  S  E  Q  L  L  N  K  L  A  P
2701  gacatagaacagatggtcggtgctgatcaaatggaagatatggaacgtatagctgtgaac
    -  D  I  E  Q  M  V  G  A  D  Q  M  E  D  M  E  R  I  A  V  N
2761  atgagtaaaatggtgtttgttggcaacgtagcttcaggatttagtccagctatagcaagt
    -  M  S  K  M  V  F  V  G  N  V  A  S  G  F  S  P  A  I  A  S
2821  aaaatggtagaatctacaaatttgagaaaggtacgagcaagaaggacaggcagggtattt
    -  K  M  V  E  S  T  N  L  R  K  V  R  A  R  R  T  G  R  V  F
2881  gccgttgataatacatcgcctttaacgatagatgatgttaggaaagatcttgatttatgg
    -  A  V  D  N  T  S  P  L  T  I  D  D  V  R  K  D  L  D  L  W
2941  caccctacgattgaagatattaagaaggtaattggtgactatagttcgatgagtcttttg
    -  H  P  T  I  E  D  I  K  K  V  I  G  D  Y  S  S  M  S  L  L
3001  actaaaccaacaagtcatgaaactttattgatgaagctaagcgtcaaacataatgtaaaa
    -  T  K  P  T  S  H  E  T  L  L  M  K  L  S  V  K  H  N  V  K
3061  ttatacagtaggttacattatatgttgatggctgagccggacataacaggtctgggacca
    -  L  Y  S  R  L  H  Y  M  L  M  A  E  P  D  I  T  G  L  G  P
3121  atattggcaactgaagattattacaaggatcttttgatattatcttttctgaccgcagaa
    -  I  L  A  T  E  D  Y  Y  K  D  L  L  I  L  S  F  L  T  A  E
3181  aaactgaatgctagaggagtaagtcagaagttctcagattatgcggtagctatggcaatg
    -  K  L  N  A  R  G  V  S  Q  K  F  S  D  Y  A  V  A  M  A  M
3241  ggtaaaaggatgaattactaaagcgagcaatacgaatggaacaaccaaacgtacgtagcg
    -  G  K  R  M  N  Y  -
3301  tgctggttgttggtggaatgatcgaatcatacgtagtgtttcgatcgttaaattgtttaa
3361  gtgaacacgtgtagtgtgcaacttaaatggc - 3391
```

Fig. 5.3 The nucleotide sequence of 3391 nt of the plus-strand of *A. bisporus* L1 dsRNA and the deduced amino acid sequence of the longest ORF. Numbering of the nucleotides is with respect to the first determined nucleotide. The sequence represents the region with homology to conserved motifs in RdRp of dsRNA viruses are underlined and highly conserved amino acids are shown in boldface.

```
   1  -ggataagtggttagtgcaagtagaattgattaggcaacggctagttggccaaattgaat

  61  catcgaacaaaacctcaacgcaaatttcataacaacatttgaatttgttcgtgcttactt

 121  taaaacatgactacacaacaagcaagtgtaatgcggttcacaactgcctattctagtaat
    -  -  -  M  T  T  Q  Q  A  S  V  M  R  F  T  T  A  Y  S  S  N
 181  aacaaagacgcgaatgacgtggtgcctgggagtctgttgcatcaaagattggtgaagagt
    -  N  K  D  A  N  D  V  V  P  G  S  L  L  H  Q  R  L  V  K  S
 241  cgggagttagaagagcatgttacgcggaattatggtttgccagatgtgatgtactcatat
    -  R  E  L  E  E  H  V  T  R  N  Y  G  L  P  D  V  M  Y  S  Y
 301  gctggcaccttacctggtgaatacgcgaggattgggagtcaaattgagaaacagataata
    -  A  G  T  L  P  G  E  Y  A  R  I  G  S  Q  I  E  K  Q  I  I
 361  gggaagaatctatccaatatactgctggagttatgtgatggagataatatggaacgggtg
    -  G  K  N  L  S  N  I  L  L  E  L  C  D  G  D  N  M  E  R  V
 421  aagatggtgactttatgccatgatgtaatgcataataaagtgcagaagatagcagcaagt
    -  K  M  V  T  L  C  H  D  V  M  H  N  K  V  Q  K  I  A  A  S
 481  cgcacttcgtgtaagttcgaagtaagtgagcagagtgatatggaggttaaaattgaagtg
    -  R  T  S  C  K  F  E  V  S  E  Q  S  D  M  E  V  K  I  E  V
 541  gcgttcggatcggccgaagatggctaccaaacaaaggaattggtgattacttttgaaaag
    -  A  F  G  S  A  E  D  G  Y  Q  T  K  E  L  V  I  T  F  E  K
 601  gtaaatgatcagtgggcgtttgtaggtatggaggcattgaatgattttagaaaggtcaga
    -  V  N  D  Q  W  A  F  V  G  M  E  A  L  N  D  F  R  K  V  R
 661  aatatgtacagagcgcgtgcagcaaaaaaacaattgcaggagcttgcggatgatcattat
    -  N  M  Y  R  A  R  A  A  K  K  Q  L  Q  E  L  A  D  D  H  Y
 721  atacattatatacgggttgtcaatactatcttgctgacggtgatgtcttgtaagagcgca
    -  I  H  Y  I  R  V  V  N  T  I  L  L  T  V  M  S  C  K  S  A
 781  attttgccaccagttttgaatttcaatgcgaacgggcctactgggcacgaaacgtatgat
    -  I  L  P  P  V  L  N  F  N  A  N  G  P  T  G  H  E  T  Y  D
 841  aaccattattggggcgagcctcaaaggaacatgaagtttactagtttgccgacggaagcg
    -  N  H  Y  W  G  E  P  Q  R  N  M  K  F  T  S  L  P  T  E  A
 901  attgaaactgggatgctgccatacacatatctggaagagaagagtgatagacaattacag
    -  I  E  T  G  M  L  P  Y  T  Y  L  E  E  K  S  D  R  Q  L  Q
 961  ccacaggaagggtgccggatgttgttcgttaccgcggaggaaaacatatatgtgcaaggt
    -  P  Q  E  G  C  R  M  L  F  V  T  A  E  E  N  I  Y  V  Q  G
1021  gtcagtaatgatatgccggcagcggatgtaatgtgttgtttgaacaataataacgcgcat
    -  V  S  N  D  M  P  A  A  D  V  M  C  C  L  N  N  N  N  A  H
1081  gacatgactatatatgcaatgcggaataaaatgactaacgaatatactatattttacgtg
    -  D  M  T  I  Y  A  M  R  N  K  M  T  N  E  Y  T  I  F  Y  V
1141  cagactccgggctatgttagtgatgggttgcgcaacaaaataaaaactcagaagaatgta
    -  Q  T  P  G  Y  V  S  D  G  L  R  N  K  I  K  T  Q  K  N  V
1201  tatcggtgtacagcatttgacgaccagaacgacaatgagttgatggagcatttgcagcca
    -  Y  R  C  T  A  F  D  D  Q  N  D  N  E  L  M  E  H  L  Q  P
1261  acacatttcgctgcgtttatgtatacgccgataggcgcggacggcaagacgatagagacc
    -  T  H  F  A  A  F  M  Y  T  P  I  G  A  D  G  K  T  I  E  T
1321  ctggacggaacgatggtggagttggaagggttggaaccagaaagaacgaaatttataggg
    -  L  D  G  T  M  V  E  L  E  G  L  E  P  E  R  T  K  F  I  G
1381  aatgatttcaggaaagatatggcatggatgtcgtttttcgtgtcccgagtgatgtataca
    -  N  D  F  R  K  D  M  A  W  M  S  F  F  V  S  R  V  M  Y  T
1441  ggagaaaaggtagtgcataggccgcgtgatagaatcagggtgaaaacttcaatcgctgaa
    -  G  E  K  V  V  H  R  P  R  D  R  I  R  V  K  T  S  I  A  E
1501  catgaatatggtcagggtttagtgagacataagtacaaaaattgcttcaaagatggggta
    -  H  E  Y  G  Q  G  L  V  R  H  K  Y  K  N  C  F  K  D  G  V
```

```
1561  gcatcgaagtacagagcgtattcacaaggagtggcagccgtgttaagcgcgctagaggtg
    -  A  S  K  Y  R  A  Y  S  Q  G  V  A  A  V  L  S  A  L  E  V
1621  gaagttaagccaggcaggttagatttaccgttaaatgcaacagtatgtcaagatgtgttg
    -  E  V  K  P  G  R  L  D  L  P  L  N  A  T  V  C  Q  D  V  L
1681  gaggtattcatggatatggaatatatgcaatgcaagacaaatggtgaagataggatggta
    -  E  V  F  M  D  M  E  Y  M  Q  C  K  T  N  G  E  D  R  M  V
1741  ataccgtatgcagtgggcgcgatattatataaaaatggagaagcaatcagcgaagtgttt
    -  I  P  Y  A  V  G  A  I  L  Y  K  N  G  E  A  I  S  E  V  F
1801  gcgagcgtcacgaaagaggacttaataagtggaatgcccaaggtcagatcttatgtagat
    -  A  S  V  T  K  E  D  L  I  S  G  M  P  K  V  R  S  Y  V  D
1861  ccaggggtaactgatatgcatgggcaattggaaaaacgtttggccaaaatgaatacagat
    -  P  G  V  T  D  M  H  G  Q  L  E  K  R  L  A  K  M  N  T  D
1921  tgggaaagtgaagtgtcgtcaagagatgtgacaaaattgttagaaataataagagaggta
    -  W  E  S  E  V  S  S  R  D  V  T  K  L  L  E  I  I  R  E  V
1981  agtgaaaataatatcaatgtgtatgccaaaggagtgaccaatgataatatagcagtgcat
    -  S  E  N  N  I  N  V  Y  A  K  G  V  T  N  D  N  I  A  V  H
2041  ggagaaataagtgagcggccgacaagtgctctgaccgtgtataagttaggcaaaaatgct
    -  G  E  I  S  E  R  P  T  S  A  L  T  V  Y  K  L  G  K  N  A
2101  aagctgaagaaacgattaggggaagcaggcctaggtgtaggaaaactttctgactatact
    -  K  L  K  K  R  L  G  E  A  G  L  G  V  G  K  L  S  D  Y  T
2161  acagaagtggagcaccggccaatacatgaaataaagctgtttgcaagtgcgattgggttt
    -  T  E  V  E  H  R  P  I  H  E  I  K  L  F  A  S  A  I  G  F
2221  aaaagttatgatgagccggaaagaatgaatgaatcgatgtttgaggagaagatcaatgag
    -  K  S  Y  D  E  P  E  R  M  N  E  S  M  F  E  E  K  I  N  E
2281  gtgataagtggatataagtgagcataaaggtatgtaaattactatagatacgccgaacac
    -  V  I  S  G  Y  K  -
2341  gatcagtcaataactatgaataagttaatgac - 2371
```

Fig. 5.4 The nucleotide sequence of 2371 nt of the plus-strand of *A. bisporus* L5 dsRNA and the amino acid sequence of the longest ORF. Numbering of the nucleotides is with respect to the first determined nucleotide. The helicase-related similarity to Chilo iridescent virus CIV is underlined and boldface(Appendix III-5).

5.3.2 Sequence analysis L1 and L5 dsRNA of *A. bisporus* V95

The putative protein encoded by the major ORF of L1 dsRNA revealed significant homology with the region of eight conserved motifs (Bruenn, 1993) of the RNA-dependent RNA polymerase (RdRp) from dsRNA viruses of simple eukaryotes.There are underlined and labelled with the deduced amino acid sequence shown in Fig. 5.3. Of the 70 amino acid positions contained in the eight conserved motifs, dsRNA of *A. bisporus* isolated UK RdRps is identical in 36, 32, 31, 30, 30, 29 and 27 positions, respectively, to the RdRps of Helminthosporium victoriae 190S virus (Hv190SV), Ustilago maydis virus 1 (UmVH1), Saccharomyces cerevisiae virus 1 (ScVL1), Leishmania guyanesis virus 1 (LRV1), the Trichomonas vaginalis virus (TvV), the Saccharomyces cerevisiae virus (ScVLa) and Giardia lamblia virus (GlV). These result are in agreement with the previous conclusion (Bruenn, 1993;Huang and Ghabrial, 1996) that the RdRps of dsRNA viruses of simple eukaryotes are very closely related (Table 5.1).

L5 dsRNA contains one large open reading frame of 724 amino acids. The putative polypeptide has a 51% similarity exists between positions 579 to 773 with the helicase Chilo iridescent virus CIV. Iridovirus within the family *Iridoviridae* are highly pathogenic for larvae of important pest insects (dsDNA non-enveloped icosahedral virus particle) (Fig 5.4 ; Appendix III-5).

5.3.3 cDNA cloning of dsRNA of *V. fungicola and T. harzianum*

RT-PCR amplifications were carried out, first with random primers and then amplified by the second round of PCR using the same primers. At this step, one major product of the expected size and a few minor products were obtained (Fig 5.5); this is better than the result obtained by a first round RT-PCR. Subsequently, standard PCR

Table 5.1 Alignment of the amino acid sequence of *A. bisporus* virus V95 L1 dsRNA underlined in Fig.5.3 with the eight conserved motifs in RdRps of dsRNA viruses of simple eukaryotes[1]

	1		2		3		4	
AbVuk	LAGRV	75	WSQPGSATG	73	GKNASRS	55	DYANFNEQHS	53
cons	*		**		**		* ** *	
LRV1	LLGRG	59	WAANGS_HS	49	GKTRLLL	57	DYDDFNSQHT	46
TvV	LLGRG	58	WSKSGS_HY	45	GKERFIY	50	DYTDFNSQHT	43
ScVL1	LMNRG	57	WVPGGSVHS	50	GKQRAIY	52	DYDDFNSQHS	52
ScVLa	LENGV	58	IMPGGSVHS	50	GKVRALY	52	DYDDFNSQHS	52
Hv190s	LQGRY	61	WCVNGSQNA	46	GKDRAIF	55	DYDNFNSQHS	45
UmVH1	LYGRG	66	WLVSGSSAG	61	GKARAIY	55	DYPDFNSMHT	63
cons	*		**		** *		* **** *	
GlV	LLGKV	65	WGTTGSGYI	41	TKVRAVT	55	DQSNFDROPA	59
BcV			GPPGGETHM	44	TKVRGVM	52	DWSSFDSSVT	52

	5		6		7		8
AbVuk	GLLSGWRCTAYINNLINIAQY	20	TGGDDGCA	34	EFFRL	9	SVIR
cons	* ** * * * *		***		* *		*
LRV1	TLMSGHRATSFINSVLNRAYI	11	HVGDDILM	33	EFLRV	9	VLAR
TvV	TLPSGHRATTFINPVLNWCYI	11	CAGDDVIL	31	EFLRK	9	VPCR
ScVL1	TLLSGWRLTTFMNTVLNWAYM	15	HMGDDVMI	33	EFLRV	13	YLSR
ScVLa	TLFSGWRLTTFFNTALNYCYL	13	HNGDDVFA	33	EFLRV	11	YLTR
Hv190s	TLMSGHRATTFTNSVLNAAYI	15	HAGDDVYL	34	EFLRL	9	YLCR
UmVH1	GLYSGDRDTTLINTLLNIAYA	20	CHGDDIIT	34	EYLRI	10	CLAR
cons	* ** * * * ** *		***		* **		* *
GlV	GLPSGWKWTALLGALINTQLL	16	VQGDDIAL	33	EFLRR	13	MMIR
BcV	GLPSGSYYTSIVGSVVNRLRI	16	TQGDDSLI	35	TFLGR	9	SLDR

[1]Data compiled from Bruenn ,1993 and Huang and Ghabrial, 1996. Residues identical in all sequences are indicated by asterisks. Virus abbreviations : the *Agaricus bisporus* virusV95 uk isolate (AbVuk), the *Leishmania guyanesis* virus 1 (LRV1), the *Trichomonas vaginalis* virus (TvV), the *Saccharomyces cerevisiae* virus 1 (ScVL1), the *Saccharomyces cerevisiae* virus (ScVLa), the *Helminthosporium victoriae* 190S virus (Hv190SV) the *Ustilago maydis* virus 1 (UmVH1), the *Giardia lamblia* virus (GlV), the beet cryptic virus (BcV).

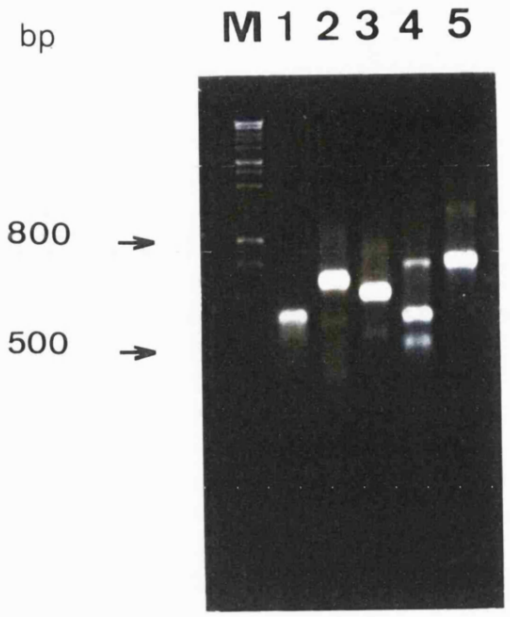


Fig. 5.5 The second round of PCR product targeting the dsRNA of *T.harzianum* T7 (lane 1, 2, 3; primer Tr2, Tf1, Tf2) and *V. fungicola* V7-3 (V; lane 4, 5 ; primer Vr2, Vf2).
Shown are the DNA amplification products.
M = Molecular weight marker (λ-DNA 100-bp ladder).

cloning was performed. Analysis of the subclones by restriction digestion with *Eco*R I yielded cDNA inserts ranging in size from about 400 bp to 800 bp (Fig. 5.6).

Clones were demonstrated to represent dsRNA sequences by hybridization to Northern blots. The Northen blot analysis indicated that individual clones hybridized to only one of the fragments, suggesting that each fragment was genetically unique (Fig. 5.7). Three of the cDNA clones from *V. fungicola* V7-3 dsRNA that were specific to dsRNA 1 of V7-3 contained inserts of v1/1 and v1/2 of approximately 1.7 kb (Fig 5.8 A). Four of cDNA clones from *T. harzianum* T7 dsRNA that were specific to dsRNA 2 of T7 contained inserts of approximately 910 bp and dsRNA 3 contained inserts of about 825 bp (Fig 5.8 B,C). The consensus sequences were determined from overlapping, independent cDNA clones (Fig. 5.9, Fig 5.10, Fig 5.11, Fig 5.12).

5.3.4 Sequence analysis of dsRNA of *V. fungicola and T. harzianum*

No extensive sequence similarity was found between the nucleotide sequences of dsRNA of *V. fungicola* isolate V7-3 or dsRNA of *T. harzianum* isolate T7 in the available databases. However, comparison of the predicted amino acid sequence of the partial dsRNA-1 of *V. fungicola* V7-3 (v1/2) revealed a striking 47% similarity with positions 667 to 918 of polyprotein RdRp of navel orange infectious mottling virus (+ssRNA comovirus, bipartite icosahedral capsids, 28 nm diameter, Appendix III-6).

There is 45% identity between the position 532 to 915 with non-structural polyprotein contains : RNA-directed RNA polymerthiol protease ; helicase (2C-like helicase/ cysteine protease) ; RdRp of San Miguel sea lion virus (+ssRNA calicivirus , isometric particles, Appendix III-6) (Fig 5.10).

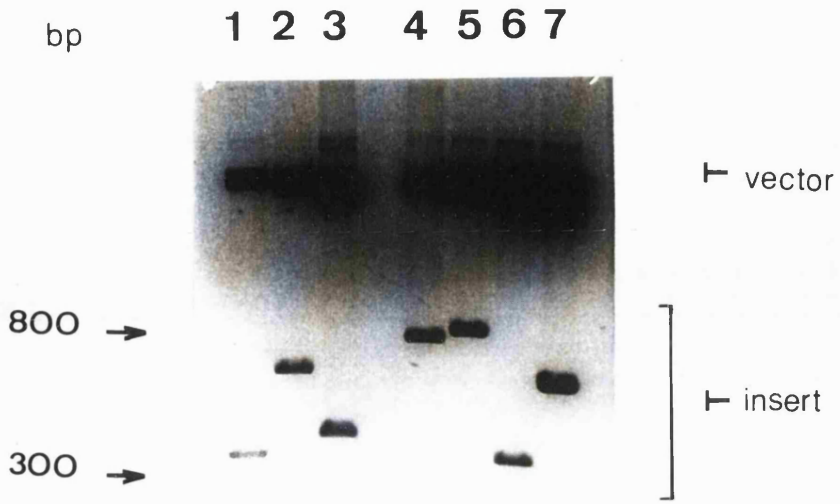


Fig. 5.6 The subclones demonstrate inserts of the expected size by restriction digests. After the second round of PCR, the expected band was excised and purified. The DNA was ligated into a T-tail cloning pGEM-T Easy vector and used to transform competent cells. Minipreps were performed, and the plasmid-DNA was digested with *Eco* RI and size fractionated on a 1% agarose gel.

Lane 1,2,3 (clone : Tr23, Tr24, Tf31) show cDNA from dsRNA of *T. harzianum* isolate T7.

Lane 4,5,6,7 (clone : Vf24, Vr21, V62, V63) show cDNA from dsRNA of *V. fungicola* isolate V7-3.

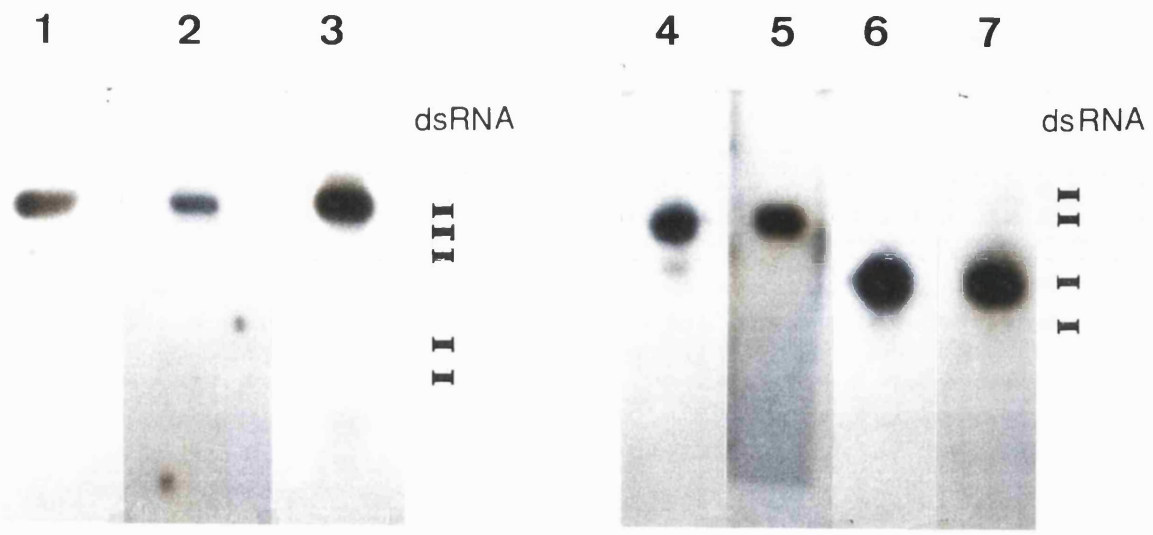


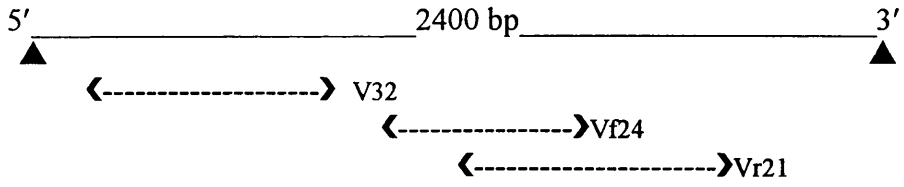
Fig. 5.7 Autoradiograph of Northern hybridization for assignment of cDNA clones to dsRNA segments. Total dsRNA of *T. harzianum* T7 (T) and *V. fungicola* V7-3 (V) were separated through a 0.8 % agarose gel, blotted to nylon membrane and hybridized with ^{32}P-labelled cDNA clones :

Lane 1, 2, 3 : clone #V32 (700 bp insert) , #Vr21 (700 bp insert), #Vf24 (600 bp insert) from dsRNA of *V. fungicola* isolate V7-3.

Lane 4, 5, 6, 7 : clone #T421(700 bp insert) , #Tr24 (600 bp insert), #T31 (700 bp insert) , #Tf31 (700 bp insert) from the dsRNA of *T. harzianum* isolate T7.

Migrating position of dsRNA are indicated at the right.

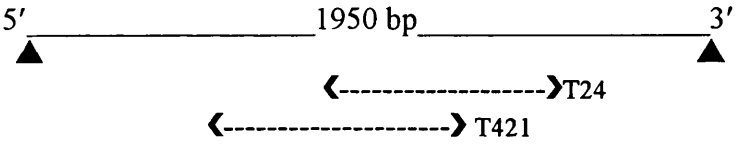
A) dsRNA 1of *V. fungicola* isolate V7-3 :



consensus: «_____712_____» «_____988_____»

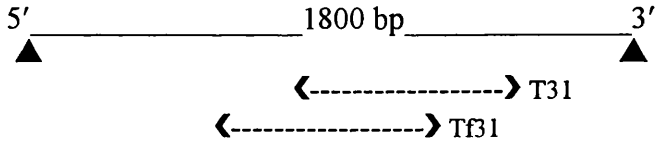
v1/1 **v 1/2**

B) dsRNA 2 of *T. harzianum* isolate T7:



consensus « _____910_____»

C) dsRNA 3 of *T. harzianum* isolate T7:



consensus « _____825_____»

Fig. 5.8 Schematic diagram showing an overlapping map of cDNA clones representing dsRNA-1of *V. fungicola* isolate V7-3 and dsRNA-2, dsRNA-3 of *T. harzianum* isolate T7. Maps are shown using the sequences in Figs 5.9, 5.10, 5.11 and 5.12 as the positive strands.

```
  1  tagacgaacagaccggccgttcggttacgggagaactgcctcccgatctggtggaccacg
   -  -  T  N  R  P  A  V  R  L  R  E  N  C  L  P  I  W  W  T  T
 61  ggcgtcaggcccagagagaaaggactggaggcgcccgagagcatccgagattgacgagta
   -  G  V  R  P  R  E  K  G  L  E  A  P  E  S  I  R  D  -  R  V
121  ccccgcgtcacgcaacacgtcatagacggcgaactccatagcggactgggcagcatgccg
   -  P  R  V  T  Q  H  V  I  D  G  E  L  H  S  G  L  G  S  M  P
181  ggcagcgccatggcgactgagagcttcatcgcccgtcgtcacagaccagcacgtccggag
   -  G  S  A  M  A  T  E  S  F  I  A  R  R  H  R  P  A  R  P  E
241  aaaggggttgtcggcgagacacatgtcacgcatgagggtggcagagacatcaagggatgc
   -  K  G  V  V  G  E  T  H  V  T  H  E  G  G  R  D  I  K  G  C
301  gtgatccatgtctcggagagtggccttgaggcactcaaggctggggaaaggttgaggaaa
   -  V  I  H  V  S  E  S  G  L  E  A  L  K  A  G  E  R  L  R  K
361  ctttccttcagccttgagtgcctcactcagccaactacagtgacattagctacagcagca
   -  L  S  F  S  L  E  C  L  T  Q  P  T  T  V  T  L  A  T  A  A
421  tcggcatcagagccgcgatcgctaatccccgcagtcgtccccgccagcatcgccgccatt
   -  S  A  S  E  P  R  S  L  I  P  A  V  V  P  A  S  I  A  A  I
481  gcttctttattcttcccccgacgactcgtccagctatccggccacctcctcacccgagcg
   -  A  S  L  F  F  P  R  R  L  V  Q  L  S  G  H  L  L  T  R  A
541  tcgtcatcctacaacatggtcgtctcccctcgctacaagcgcgagctccagatcgctgag
   -  S  S  S  Y  N  M  V  V  S  P  R  Y  K  R  E  L  Q  I  A  E
601  ctggccgtccagcgtgccgccatcctcaccaagcgcgtcttccacgaaaaggccaagggc
   -  L  A  V  Q  R  A  A  I  L  T  K  R  V  F  H  E  K  A  K  G
661  accgtctacaagaacgacaagtcacccgtcactattggtgacttcggcgctc -712
   -  T  V  Y  K  N  D  K  S  P  V  T  I  G  D  F  G  A
```

Fig. 5.9 The partial nucleotide sequence of 712 bp (v1/1) of the plus-strand dsRNA-1 of *V. fungicola* isolate V7-3 and deduced amino acid sequences.

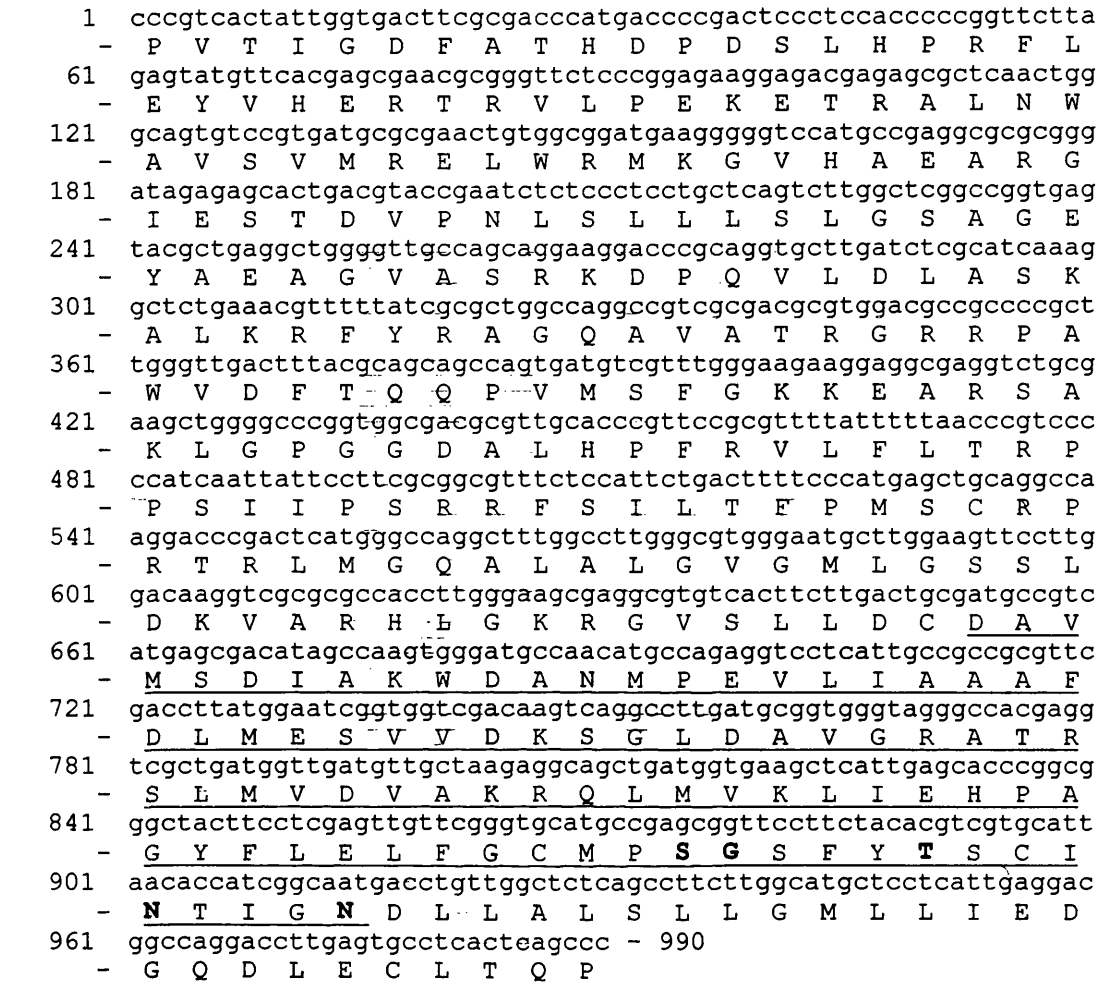


Fig. 5.10 The partial nucleotide sequence of 990 bp (v1/2) of the plus-strand dsRNA-1 of *V. fungicola* isolate V7-3 and amino acid sequences of the regions are shown and the conserved amino acid sequence RdRp motif is underlined and highly conserved amino acid are shown in boldface (Appendix III-6).

```
  1  tgcatcgtccgggccgcccatgtcgcccggcggaattcgattgggcttgttaacactttg
   -  C  I  V  R  A  A  H  V  A  R  R  N  S  I  G  L  V  N  T  L
 61  gaagccggactggctattgtacttcccgtttgttttccattatttgctcacttaattcca
   -  E  A  G  L  A  I  V  L  P  V  C  F  P  L  F  A  H  L  I  P
121  aagaattatatctcacccttaagagatacagtttgggaataccgctactttgacgacgct
   -  K  N  Y  I  S  P  L  R  D  T  V  W  E  Y  R  Y  F  D  D  A
181  gtcaaaggcactattcgccaaccaaattcagttggatcttctgaacatttatacatggat
   -  V  K  G  T  I  R  Q  P  N  S  V  G  S  S  E  H  L  Y  M  D
241  aatcgttttattgaatgcatccgcgacttaaatttcggtcgtttaaaaagagtgcaacaa
   -  N  R  F  I  E  C  I  R  D  L  N  F  G  R  L  K  R  V  Q  Q
301  gaacgttctgaggagaaaatcgataaagtcgaacagccgacattaactcgtctatctcaa
   -  E  R  S  E  E  K  I  D  K  V  E  Q  P  T  L  T  R  L  S  Q
361  gaagatatgcgacgacgcttacgtaaagcacataagtcggctaatttacgagagttcacc
   -  E  D  M  R  R  R  L  R  K  A  H  K  S  A  N  L  R  E  F  T
421  agacaaccatccttcccgctacccatgcgtattaccactggtatgtccgatcacccaata
   -  R  Q  P  S  F  P  L  P  M  R  I  T  T  G  M  S  D  H  P  I
481  cacgaacctctagacgtcgaaccgactaacacagcacctgttacgccagttcttcgaact
   -  H  E  P  L  D  V  E  P  T  N  T  A  P  V  T  P  V  L  R  T
541  tcagttaaatcaatgactttcgattcaccacctgttattccaacatcttctgagcctaaa
   -  S  V  K  S  M  T  F  D  S  P  P  V  I  P  T  S  S  E  P  K
601  gctacagctgtttcaactacagttagctccattactacagcggcttctaaagtgttaaca
   -  A  T  A  V  S  T  T  V  S  S  I  T  T  A  A  S  K  V  L  T
661  gcccttgctaaacaaagctttggcgctaatgctactattggatcaacagaacccgatgac
   -  A  L  A  K  Q  S  F  G  A  N  A  T  I  G  S  T  E  P  D  D
721  acggactatattccagatcctatcttaaactttcaaatgagagactttgtttctcatatc
   -  T  D  Y  I  P  D  P  I  L  N  F  Q  M  R  D  F  V  S  H  I
781  aaaggtttagatgagatgccattgcatcttgatcacatggaaagattcgcttgcgactac
   -  K  G  L  D  E  M  P  L  H  L  D  H  M  E  R  F  A  C  D  Y
841  gagacaccttggcctctaaccagactacaagttagacctaatgatgctcgtgcccttcaa
   -  E  T  P  W  P  L  T  R  L  Q  V  R  P  N  D  A  R  A  L  Q
901  cgcagctcgc - 910
      R  S  S
```

Fig. 5.11 The partial nucleotide sequence of 910 bp of the plus-strand dsRNA-2 of *T. harzianum* isolate T7 and putative amino acid sequences of the regions are shown. The similar amino acid sequence with hypothetical 69 kD protein of turnip yellow mosaic virus is underlined and boldface (Appendix III-7).

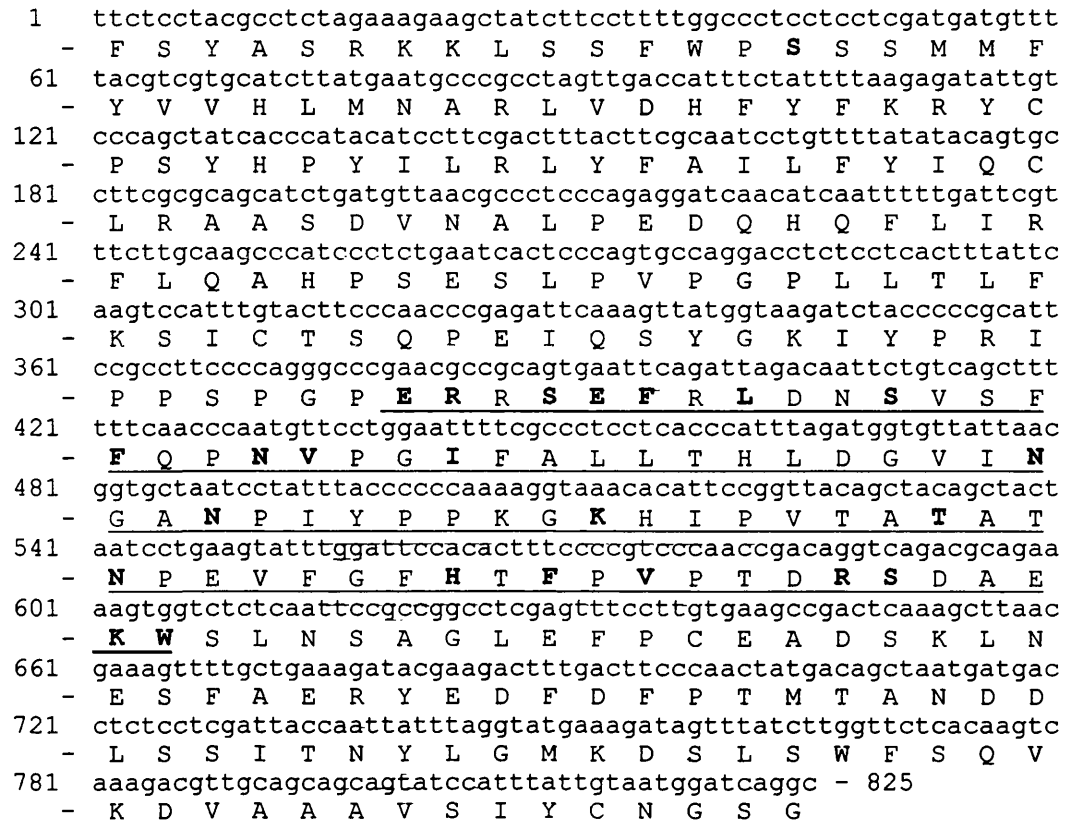


Fig. 5.12 The partial nucleotide sequence of 825 bp of the plus-strand dsRNA-3 of *T. harzianum* isolate T7 and amino acid sequences of the regions are shown. The region with 41% similarity with the guanine nucleotide-binding protein alpha-3 subunit of *Ustilago maydis* is underlined and boldface (Appendix III-8).

The putative amino acid sequence encoded by dsRNA-2 of *T. harzianum* isolate T7 revealed 46 % identity with a hypothetical 69 kDa protein of turnip yellow mosaic virus (+ssRNA tymovirus , icosahedral capsid, 29 nm diameter) that was found at amino acid position 76 - 264 (Fig 5.11).

The amino acid sequences deduced from the partial nucleotide sequences of dsRNA-3 of *T. harzianum* isolate T7 has a 41% similarity between positions 1 - 444 with amphiphysin a protein of synaptic vesicles , that is believed to be involved in the synaptic vesicle recycling ; Appendix III-8 (Munn, *et al.*, 1995). At position 379 - 606, there was 41% similarity with the guanine nucleotide-binding protein alpha-3 subunit of *Ustilago maydis* (Fig 5.12).

5.3.5 Interrelationships among dsRNAs isolates

A. bisporus from the UK and EU

Hybridization analyses of the two isolates V95 and EU was carried out using ^{32}P-labeled cDNA of dsRNA L1 from *A. bisporus* isolate V95. The two isolates had L1 and L5 dsRNA bands of the same molecular weight as shown by electrophoresis analysis (Fig. 5.13 A). The L1 dsRNA of V95 cross-hybridized with L1 dsRNA of EU suggesting that their dsRNAs are closely related (Fig 5.13 B). The L5 dsRNA of *A. bisporus* isolate V95 cross-hybridized with L5 dsRNA of isolate EU (Fig. 5.13C). The hybridization results indicate sequence homology between isolate V95 and EU.

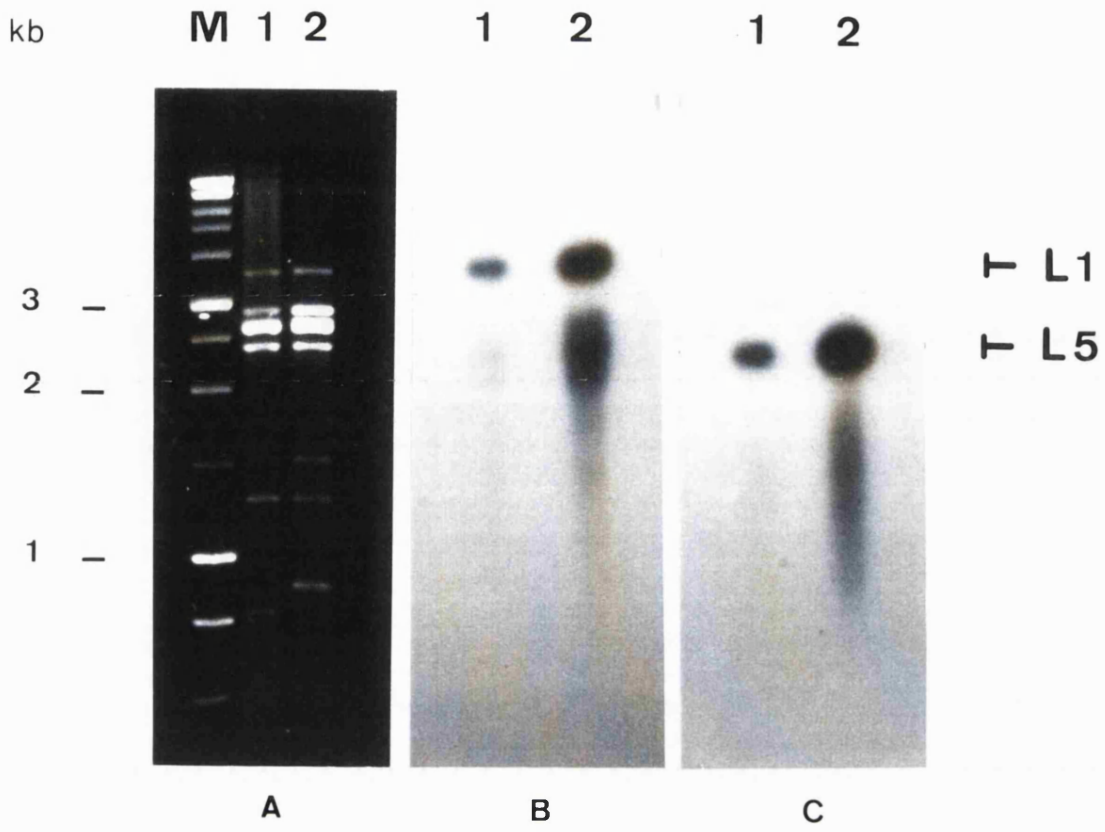


Fig. 5.13 Agarose gel electrophoresis and Northern blot analysis.

A= dsRNA from *A. bisporus* two isolates.

Lane M = Molecular weight marker (kb DNA Ladder)

Lane 1 = *A. bisporus* isolate V95 from UK.

Lane 2 = *A. bisporus* isolate EU.

B = Autoradiograph of dsRNA using the ^{32}P-labeled cDNA clone from *A. bisporus* (V95) corresponding to the position of dsRNA 1(1000 bp) as probe.

C= Autoradiograph of dsRNA using the ^{32}P-labeled cDNA clone from *A. bisporus* (V95) corresponding to the position of dsRNA 5 (700 bp) as probe.

***T. harzianum* from England, Northern Ireland and Republic of Ireland**

The interrelationships between dsRNA of *T. harzianum* group I : isolates T7, KPNT, T32, Th1c and group II : isolates Th3c, A006022 were determined by hybridization technique and probing with a ^{32}P-labeled cDNA of dsRNA 2 from *T. harzianum* isolate T7. The result (Fig. 5.14 B) indicated that this probe hybridized with dsRNA from the same group I :T7, KPNT, T32, Th1c but not with dsRNA from group II :Th3c, A006022. Even though both groups showed electrophoretic banding pattern of dsRNA 2 of the same size (Fig. 5.14 A), they did not show significant sequence homology in the hybridization assay suggesting that they may be part of a different viruses or virus complex.

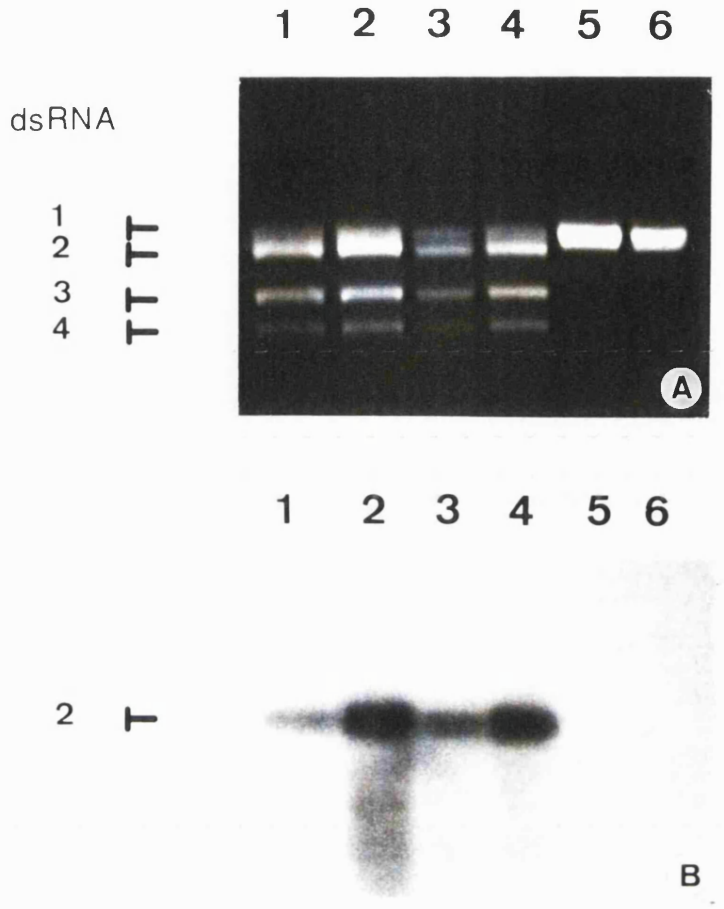


Fig. 5.14 Agarose gel electrophoresis and Northern blot analysis.

A = dsRNA from *T. harzianum* six isolates.

Lane 1 = T7; England Lane 2 = KPNT; England

Lane 3 = T32; England Lane 4 = Th1C; Northern Ireland

Lane 5 = Th3C; Northern Ireland Lane 6 = A006022; Rep. of Ireland

B = Autoradiograph of dsRNA using the ^{32}P-labeled cDNA clone from *T. harzianum* isolate T7 corresponding to the position of dsRNA 2 (700 bp) as probe.

5.4 DISCUSSION

In a previous report (Van der Lende, *et al.*, 1996) , a number of cDNA clones of L1 and L5 dsRNA in *A. bisporus* from The Netherlands were isolated from the cDNA library constructed by Harmsen, *et al.*, (1991) and identified by hybridization with individually isolated and α^{32}P-dCTP-labeled dsRNAs. None of these cDNAs appeared to be full-length but together the clones could represent all sequences present because 3′ends of both strands of the dsRNAs were used in the initial polyadenylation reaction. In this study, using primers based on published sequences , a series of clones was made through RT-PCR mediated amplification from total dsRNA of *A. bisporus* isolate V95 and then sequenced in their entirety. The present results demonstrate that rapid cloning of the dsRNA gene can be achieved directly from a small amount of total dsRNA using RT-PCR. The current procedure makes it possible to omit the complicated and many time-consuming steps. However, RT-PCR is dependent upon the quality of the template RNA, duration of cDNA synthesis reaction, and optimization of the PCR protocol for each target. The present RT-PCR procedure could also be applied to rapid cDNA cloning of other dsRNA viruses if the nucleotide sequence of both terminal portions of expected gene is available.

In the case of cDNA clones of dsRNA of *V. fungicola* isolate V7-3 and *T. harzianum* isolate T7, these are newly described dsRNA-viruses whose sequences have not yet been reported. A partial nucleotide sequence was derived by using a randomly primed RT-PCR protocol. The 5′ RACE (Rapid Amplification of cDNA Ends) which allows the amplification of unknown sequences at the 5′end of the RNA could be used to complete the sequences, in furture work. The procedure is a rapid, sensitive method for the 5′ end of a particular mRNA. The sequences at the 5′- termini of dsRNA can be determined from partial cDNA clones and poly (A) can be added to both 3′ termini of dsRNA then subjected to RT-PCR in the presence of oligo (dT) and an internal specific primer (Frohman, *et al.*, 1988).

Due to the recent developments of molecular techniques, it is possible to detect viruses using nucleic acid hybridization (Hunst, *et al.*, 1986) or RT-PCR (Sonnenberg, *et al.*, 1995 b). PCR especially has been used successfully to detect infectious human viruses (Kwok, *et al.*, 1987), plant viruses (Rybicki and Hughes, 1990 ; Vunsh, *et al.*, 1990 ; Takamatsu, *et al.*, 1994), dsRNA virus (Smith, *et al.*, 1992), viroids (Hayata, *et al.*, 1992), plant pathogenic bacteria (Dong, *et al.*, 1992) or fungi (Schesser, *et al.*, 1991), because it offers the following advantages: 1) only a small fragment of a material is sufficient to detect the virus, 2) detection by PCR is more sensitive than other conventional methods so far used.

Harmsen (1990) described a RT-PCR method for detecting the presence of dsRNAs in spawn-run compost. Although it was shown that RT-PCR is in principle applicable in compost, results were not consistent, probably due to inhibitory compounds present in compost extracts. By optimizing several parameters, Sonnenberg, *et al.*, (1995 b) have shown that RT-PCR can be used as a reliable test for the presence of La France disease in spawn-run compost. It is especially important to test at least two dilutions of each compost extract in a range equivalent to 0.5-5 μg freeze-dried compost per RT-PCR reaction. Low dilutions of samples inhibited the RT-PCR by the presence of inhibitory compounds and high dilutions lowered the concentration of dsRNAs beyond the detection limit. Testing of more than one dilution of each sample is also important because the concentration of the unknown inhibitory compounds in compost extracts may vary from sample to sample. In addition, the amount of mycelial biomass also varies in spawnrun compost samples.

The nucleotide sequence data showed that the putative protein encoded by the major ORF of L1 dsRNA of *A. bisporus* V95 revealed significant homology with the region of eight conserved motifs (Bruenn, 1993) of RNA-dependent RNA polymerase (RdRp) from dsRNA viruses of simple eukaryotes in current protein databases. The partial putative protein of L5 dsRNA of *A. bisporus* V95 has a 51% similarity to the helicase Chilo insect iridescent virus (CIV). This is the type species of the genus Iridovirus within the family Iridoviridae and is highly pathogenic for larvae of important pest insects (Bahr, *et al.*, 1997 and Muller, *et al.*, 1999).

Sonntag, *et al.*, 1994 reported that a 606 codon open reading frame (ORF) of CIV located in this region encoded a protein (p69) related to a distinct family of putative DNA and/or RNA helicases belonging to the "DEAD/H" superfamily. Unique sequence signatures were obtained that allowed selective retrieval of the putative helicases of the new family from amino acid sequence databases. The family includes yeast, *Drosophila*, mammalian, and bacterial proteins involved in transcription, regulation and repair of damaged DNA. It is hypothesized that p69 of CIV may be a DNA or RNA helicase possibly involved in viral transcription. A distant relationship was observed to exist between this family of helicases and another group of proteins that consists of putative helicases of poxviruses, African swine fever virus and yeast mitochondrial plasmids. It is shown that p69 of CIV is much more closely related to cellular helicases than any of the other known viral helicases. Phylogenetic analysis suggested an independent origin for the p69 gene and the genes encoding other viral helicases.

In a previous study (Harmsen, *et al.*, 1991 and Van der Lende, *et al.*, 1996), the nucleotide sequences of five of the dsRNA indicated that L3 encoded the 90 kDa major capsid polypeptide, L1 encode the 115 kDa virion-associated RdRp, and L5, M1 and M2 each encoded single proteins of unknown function. The RdRp amino acid sequence had motifs showing some resemblance to those of totiviruses, but the genome organization of this virus is clearly different from totiviruses (Buck, 1998). Further investigation will be required to determine if the complex of nine dsRNA segments represents one or more viruses and whether some of the dsRNA could be satellites.

The partial sequence of dsRNA-1 in *V. fungicola* V7-3 contained at least the RNA helicase (2C like protein) and RdRp in the same fragment, required for the replication and transcription of these virus-like genetic elements. At least one conserved motif of all RdRps showing RNA template specificity was found in the partial sequence of dsRNA-1 in V7-3. In a general sense, the genomic organization and replication strategy of the RNA viruses is reflected in the similarities among their polymerases. The positive strand viruses form one easily recognized class, the dsRNA viruses another, the negative strand RNA viruses a third, and the retroviruses a fourth

(Poch, *et al.*, 1989). The positive strand and the dsRNA viruses have recognizably similar RdRps sequences, whereas the negative strand RNA viruses and retroviruses are both distinctly different. This corresponds with suggestions that RNA viruses represent several independent lines of evolution (Bruenn, 1991).

In general, the relationships among RdRps follow the division of prokaryotic and eukaryotic hosts, but not the separation of plant, animal, and fungal cells. One large cluster of viruses includes those that infect plants, vertebrates, and/or insects. This may be the result of specialization of insect viruses to infect host species on which insects are pathogens. All the positive strand RNA viruses of this supergroup may have evolved from an ancestral dsRNA virus (Bruenn, 1991).

Jian, *et al.*, (1998) have shown significant amino acid sequence similarity of the 6.4 kb M1 dsRNA from *Rhizoctonia solani* ,with the 1A protein of broad bean mottle virus (BBMV) and other bromoviruses, that included six conserved helicase domains and an ATP/GTP binding motif. The conserved NTP-binding site motif is G/AXXXXGKS/T (Walker, *et al.*, 1982). The biochemical function for the ATP/GTP binding activity would be to supply energy or substrate for RNA polymerization (Dzianott and Bujarski, 1991). Another function for NTP-binding might be related to the NTP-dependent dsRNA unwinding (helicase) activity that operates during dsRNA replication (Hodgman, 1988).

The presence of the helicase domains and the NTP-binding motif in the sequence suggests that the putative polypeptide is involved in the replication of dsRNA. All six conserved, helicase-related , amino acid domains of bromoviruses are present in M1. The amino acid sequence similarity between M1 and bromoviruses is limited to one gene in contrast to the relationship between the *Cryphonectria parasitica* hypovirus and the (+) ssRNA potyviruses, which appears to include genomic organization (Koonin, *et al.*, 1991). Haseloff and coworkers (1984), however, reported striking similarities in amino acid sequence among nonstructural protein encoded by RNA viruses that differ in genomic organization and viral gene expression. Their study included the plant virus groups cucumoviruses, bromoviruses,

and tobamoviruses and the animal alphaviruses. They argued that the most plausible explanation for these phylogenies is that " similar genes may be incorporated independently into different viral genomes from a separate common source, presumably cellular genes"

The putative amino acid sequence encoded in the partial sequence dsRNA-2 of *T. harzianum* isolate T7 revealed a 46% similarity with a hypothetical 69 kDa protein of turnip yellow mosaic virus. Seron, *et al.*,(1996) reported that the nonstructural 69 kDa protein of turnip yellow mosaic virus is necessary for systemic spread of the virus within the plant. To examine the behavior of the 69K protein *in vivo*, the full-length 69 kDa protein was also expressed in insect cells using a recombinant baculovirus. Studies on the post-translational modifications of the 69 kDa protein in insect cells revealed that the protein is phosphorylated but not glycosylated. Further experiments on the subcellular fraction and indirect immunolocalization in insect cells showed that the 69 kDa protein is localized in the cytoplasm and/or in the plasma membrane.

The amino acid sequences deduced from the nucleotide sequences of partial dsRNA-3 of *T. harzianum* isolate T7 has a 41% similarity with amphiphysin I ; this protein of synaptic vesicles , is believed to be involved in the synaptic vesicle recycling. A role of amphiphysin I in endocytosis is further suggested by genetic studies in the yeast *Saccharomyces cerevisiae*. Disruption of the genes encoding the homologue of amphiphysin, Rvs167, or the closely related protein Rvs161, produces a potent inhibition both of receptor-mediated and fluid phase endocytosis (Munn, *et al.*, 1995). Mutation of the RVS161 and RVS167 genes also produces other pleotropic effects including actin defects (Balguerie, *et al.*, 1999). The latter phenotype is consistent with the general link between actin function and endocytosis, which has emerged from genetic studies in yeast (Munn, *et al.*, 1995 and Sivadon, *et al.*, 1995). Amphiphysin I contains many putative phosphorylation sites. Bauerfeind, *et al.*, (1997) assumed that amphiphysin I is a phosphoprotein that undergoes stimulation dependent dephosphorylation in parallel with its binding protein, dynamin I and synaptojanin I.

Position 379 - 606 of the partialy sequenced dsRNA-3 of *T. harzianum* isolate T7 showed 41% similarity with the guanine nucleotide-binding protein alpha-3 subunit of *Ustilago maydis*. This protein might be the cause of down-regulation of G protein (GTP-binding protein) production in the host fungus. These results support the effect of dsRNA on conidiation of *T. harzianum* T7h cultures which was increased about 63% in its ability to conidiate in heat cured cultures when compared to dsRNA-containing T7.

Infection of *C. parasitica* with hypovirus dsRNA caused hypovirulence and a variety of other effects, such as reduction of orange pigmentation, reduced conidiation, loss of female fertility, down-regulation of the production of oxalate, laccase (a polyphenol oxidase), cryparin (a cell surface hydrophobin), putative mating-type A pheromones, cellulase (including a cloned cellobiohydrolase I), cutinase, protease (endothiapepsin), polygalacturonase and the α-subunit of a G protein (GTP-binding protein, CPG-1), and up regulation of cyclic AMP levels. Several of these effects may be related to hypovirulence, e.g. down-regulation of cellulase, which is normally induced in the virulent plant pathogen interaction and is likely to be required for plant cell wall hydrolysis. Down-regulation of G protein production appears to be central to the hypovirulent phenotype. G proteins are known to be involved in a number of signal transduction pathways in mammalian cells and probably function by negatively regulating adenyl cyclase. Down-regulation of G protein production in *C. parasitica* is consistent with an increase in levels of cyclic AMP and with the hypothesis that signal transduction pathways, normally induced in *C. parasitica* by its interaction with the chestnut tree host, are suppressed (Buck, 1998).

Choi, *et al.*, (1995) reported that strains of the chestnut blight fungus *C. parasitica* harboring RNA viruses of the genus Hypovirus exhibit significantly reduced levels of virulence (called hypovirulence). The accumulation of a heterotrimeric GTP-binding protein (G protein) alpha subunit of the Gi class was found to be reduced in hypovirus-containing *C. parasitica* strains. Transgenic cosuppression, a phenomenon frequently observed in transgenic plants, reduced the accumulation of this alpha subunit in virus-free fungal strains. Significantly, the resulting transgenic fungal strains were also hypovirulent. These results indicate a crucial role for

G-protein-like signal transduction in fungal pathogenesis and suggest a molecular basis for virus-mediated attenuation of fungal virulence.

Kasahara and Nuss, (1997) reported that targeted disruption of two G-protein alpha subunit genes in the chestnut blight fungus *C. parasitica* revealed roles for the Gi alpha subunit CPG-1 in fungal reproduction, virulence, and vegetative growth. A second G alpha subunit, CPG-2, was found to be dispensable for these functions. They report the cloning and targeted disruption of a *C. parasitica* G-protein beta subunit gene. The deduced amino acid sequence encoded by this gene, designated cpgd-1, was found to share 66.2, 65.9 and 66.7% amino acid identify with G beta homologues from human, Drosophila and *Dictyostelium origins* , respectively, but only 39.7% identity with the *Saccharomyces cerevisiae* G beta homologue STE4 product. Low stringency Southern hybridization failed to detect any related G beta subunit genes in *C. parasitica*. Targeted disruption of cpgb-1 resulted in several of the changes previously reported to accompany disruption of the *C. parasitica* Gi alpha subunit gene cpg-1. These included very significant reduction in pigmentation, asexual sporulation, and virulence. In contrast to results obtained for Gi alpha gene disruption, the reduction in virulence resulting from the disruption of a G beta gene was accompanied by increased, rather than decreased, vegetative growth on synthetic medium. The relevance of these results to mechanisms of fungal virulence is considered.

Kruger, *et al.*, (1998) found that the phytopathogenic basidiomycete *U. maydis* mating and dikaryon formation are controlled by a pheromone/ receptor system and the multiallelic b locus. Recently, a gene encoding a G protein alpha subunit, gpa3, was isolated and has subsequently been implicated in pheromone signal transduction. Mutants deleted for gpa3 are sterile and nonpathogenic, and exhibit a morphology that is similar to that of mutants with defects in the adenylate cyclase gene uac1. They have found that the sterility and mutant morphology of gpa3 detection strains can be rescued by exogenous cAMP. In these mutants and in the corresponding wild-type strains, exogenous cAMP stimulates pheromone gene expression to a level comparable to that seen in the pheromone-stimulated state. In addition, they demonstrate that uac1 is epistatic to gpa3. They conclude that gpa3 controls the cAMP signaling pathway in *U. maydis*.

Recently, three G protein alpha subunit genes have been cloned and characterized from *Magnaporthe grisea*: magA is very similar to CPG-2 of *C. parasitica* ; magB is virtually identical to CPG-1 of *C. parasitica* , to gna1 of *Neurospora crassa*, and to fadA of *Emericella nidulans*; and magC is most similar to gna2 of *N. crassa*. Homologous recombination resulting in targeted deletion of magA had no effect on vegetative growth, conidiation, or appressorium formation. Deletion of magC reduced conidiation, but did not affect vegetative growth or appressorium formation. However, disruption of magB significantly reduced vegetative growth, conidiation, and appressorium formation. MagB-transformants, unlike magA- and magC-transformants, exhibited a reduced ability to infect and colonize susceptible rice leaves. G protein alpha subunit genes are required for *M. grisea* mating. The magB-transformants failed to form perithecia, whereas magA- and magC- transformants did not produce mature asci. These results suggest that G protein alpha subunit genes are involved in signal transduction pathways in *M. grisea* that control vegetative growth, conidiation, conidium attachment, appressorium formation, mating and pathogenicity (Liu and Dean, 1997).

Blumenthal and Carmichael, (1979) considered that viruses with small genomes often utilize host components for their replication. For example, the RdRp of bacteriophage Qβ consists of one virus-encoded and three host-encoded subunits, and an additional host protein is needed for negative-strand synthesis on a positive-strand template. Moreover, a highly purified preparation of an RdRp from plant infected by turnip yellow mosaic virus was shown to contain one virus-encoded polypeptide and one host polypeptide, although it was not established whether the host polypeptide was needed for activity, and the reaction products were not characterized (Candresse, *et al.*, 1986).

Marienfeld, *et al.*, (1997) have recently pointed out an apparent amino acid sequence similarity between plant mitochondrial DNA, the *R. solani* M2 dsRNA, and the dsRNA from *C. parasicati* strain NB631, and suggested that (i) plant mitochondrial sequences from *Arabidopsis thaliana* and bean originated from a fungal virus and (ii) nucleic acid sequence transfers take place between fungal viruses and

plants. However, as Haseloff, *et al.*, (1984) observed, direct phylogenetic relationships, as opposed to protein sequence conservation, do not readily account for the major differences in genomic organization among viral entities from phylogenetically distant organisms.

Multiple examples exist in which dsRNA are non-encapsidated, and include the T and W dsRNAs from *Saccharomyces cerevisiae*, 2871 nt and 2505 nt respectively, that are thought to encode RdRps (Esteban, *et al.*, 1993). Another well-known example is in the chestnut blight ascomycete *C. paracitica*, in which virulence can be modulated by the presence of virus-like dsRNA genetic elements (Anagnostakis and Day, 1979). The largest L-dsRNA from *C. parasitica* (12712 nt) has two ORFs, both of which encode polyproteins that undergo autocatalytic processing ; one protein may be RdRp (Shapira, *et al.*, 1991). This dsRNA was found in membranous vesicles and lacks a protein coat. A smaller dsRNA element (2728 nt) was isolated from a moderately hypovirulent strain of *C. parasitica* and is localized in the mitochondria. The deduced amino acid sequence of this dsRNA also encodes a putative RdRp (Polashock and Hillman, 1994).

Genetic relationships between the isolates were analyzed by cDNA from dsRNA in *A. bisporus* UK isolate cross-hybridization. The dsRNA L1 and L5 in *A. bisporus* EU isolate showed high levels of cross-hybridisation. There was also evidence of significant sequence similarity between UK isolate and The Netherlands isolate. There is published sequences data for the L1 and L5 dsRNA of *A. bisporus* virus 1 isolate The Netherlands by Van der Lende, *et al.*, 1996.

Difficulties in obtaining highly purified preparations of mycoviruses have presented problems in establishing serological relationship (Ghabrial, 1980). Even when antisera have been prepared to host mycoviruses, they have been found to contain antibodies to host constituents. This has hindered studies using ELISA (enzyme linked immuno sorbent assay) for establishing mycovirus relationships (Bozarth, 1977).

Hunst, *et al.*, (1986) reported that mycelial extracts of *Pyricularia oryzae* were analyzed for dsRNA content. Two of the isolates, 455 and 793, spherical particles 35 nm in diameter were purified and found to contain dsRNAs. The differences in the dsRNAs electrophoretic profiles within the particles from the two isolates, would indicate that the viruses are distinct, albeit morphologically similar. The dot-blot hybridization analyses supported this concept, because the 455 dsRNA probe did not hybridize to the 793 dsRNA. The hybridization analyses using ^{32}P-labeled dsRNA were found useful for elucidating rudimentary relationships among some of the dsRNAs extracted from *P. oryzae* isolates. Further hybridization studies are needed to clarify relationships among dsRNA viruses isolates. For the future, hybridization analyses will be a useful method for establishing relationships.

A previous study by Lhostis, *et al.*, (1985) provided the first direct evidence that hypovirulent strains of the chestnut blight fungus *C. parasitica* from European H strains have dsRNA sequences that are distinct from those of dsRNAs from American H strains. This is not surprising in view of the known differences between these strains in phenotype and dsRNA electrophoretic patterns (Anagnostakis, 1982). The lack of cross-hybridization between dsRNA from European and American H strains suggests that they represent distinct virus-like agents rather than strains of the same agent. The dsRNA from H strains from the same continent, on the other hand, were clearly closely related despite coming from widely separated locations, e.g. Michigan and Virginia. However, since the number of strains of either American or European origin examined so far is small, it is possible that H strains whose dsRNAs are unrelated may still be found on any one continent.

The dsRNA occur in many size classes in individual hypovirulent (H) strains of the chestnut blight fungus, *C. parasitica* (Dodds, 1980 a). Thus, for an understanding of the role of dsRNA in hypovirulence, it is important to determine the relatedness of dsRNA components within a given H strain and between those of different H strains. Based on electrophoretic banding patterns, Dodds (1980 a) proposed to classify dsRNAs into three types; types I and II encompass the European H strains, and type III comprises all H strains found naturally in the United States. Unfortunately,

electrophoretic banding patterns cannot be relied upon for determining the interrelationships among dsRNA from different sources since variations in number, size and relative abundance of the electrophoretic components were observed upon repeated subculturing of H strains or transfer of dsRNA to a new strain (Dodds, 1980 b ; Anagnostakis and Day, 1979; Anagnostakis, 1981; Van Alfen, 1982).

Group I isolates of *T. harzianum* showed four dsRNA bands .Two dsRNA bands of identical electrophoretic mobility as dsRNA-1 and dsRNA-2 were found in group II isolates but there did not cross hybridize with group I dsRNA (Fig. 5.14).This finding re-emphasizes that banding patterns should not be relied upon for studying interrelationships among dsRNAs (Van Alfen, 1982). The relatedness of dsRNA components present in an individual isolate has not yet been determined but only isolate T7 has been examined for virus particles and partial genomic sequence. It is not completely understood whether the multiple components of dsRNA represent a segmented genome of a single virus or mixed infection with two or more viruses. Also, the possibility that only one dsRNA component is essential for replication and others are satellites or deletion mutants cannot be ruled out. These interrelationships need to be investigated. The lack of homology between dsRNA group I from England, Northern Ireland and group II from Northern Ireland, Republic of Ireland isolates suggested that these dsRNAs will not interfere with or exclude each other.

In conclusion, the data presented in this chapter support the hypothesis that genome organization of dsRNA of *A. bisporus* isolate V95 is different from any other known mycoviruses. The partial amino acid sequence of dsRNAs of *V. fungicola* isolate V7-3 and *T. harzianum* isolate T7 showed greater similarities to those of +ssRNA isometric viruses than dsRNA viruses or any other mycoviruses. Furthermore, sequence data may also lead to testable hypotheses regarding potential mechanism by which a particular dsRNA element may affect the biology of its fungal host. Knowledge of its genome organization and expression will be valuable for understanding its role in pathogenesis and for potential exploitation in the development of biocontrol measures.

CHAPTER 6

General discussion

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Viruses are among the variety of cytoplasmic genetic elements that have been identified in fungi. From current knowledge, it appears that , at least in one way, they have evolved differently from viruses of other organisms in that they appear to lack infectivity yet are still maintained in their hosts. The most common fungal viruses have dsRNA genomes and are recognized as viruses even though some, such as the Hypoviridae, lack protein capsids (Hillman, *et al.*, 1995). The acceptance of these genetic elements as viruses, is not without controversy, because of their lack of infectivity. These elements are transmitted in fungi in a similar manner to the transmission of plasmids in prokaryotes and, therefore, never leave a cytoplasmic environment. The biology of dsRNA fungal viruses is interesting, both because they represent an unusual adaptation of a parasite to its host and because of the variety of phenotypes that has been found to be associated with characterized dsRNAs (McCabe, *et al.*, 1999).

In this study, a number of aspects were studied including the incidence and effect of dsRNA in fungi from different geographic locations, the effect of the presence of dsRNA on physiology and cytology of virus infected fungal cells and the characterisation of VLPs and molecular aspects of the dsRNAs elements from *Agaricus bisporus* isolate V95, *Verticillium fungicola* isolate V7-3 and *Trichoderma harzianum* isolate T7. These studies describe the discovery of novel dsRNA elements in *V. fungicola* and *T. harzianum.*

An investigation into the extraction and purification of dsRNA showed that a modification of the method outlined by Jordan and Dodds (1985) was reliable quick and simple ; when results are positive, considerable information is generated for use in dsRNA virus diagnosis. The extraction procedure was carried out at room temperature to avoid cellular ssRNA binding to cellulose CF-11 at 4 °C in 16% ethanol. The dsRNA recovered was a valuable reagent that could be used for RT-PCR and cloning. It is important to determine the relatedness of dsRNAs, and to recognize that dsRNAs of similar size or mobility detected by gel electrophoresis may not have

sequence homology ,therefore, be part of different and unrelated virus or dsRNA.

Hybridization experiments of dsRNA in *T. harzianum* found that the 0.8 kb cDNA T7 probe hybridized with the dsRNA-2 band of isolates in group I (T7, KPNT, T32, Th1C from England and Northern Ireland) . No cross-hybridization was observed with dsRNA of isolates in group II (Th3C, A006022 from Northern Ireland and Rep. of Ireland). Hybridization studies are only able to provide relatively crude estimates of dsRNA diversity and population structure. However, it is necessary first to show which hybridization groups are present before finer resolution analyses can be performed. The results show that group I isolates from the different geographic origins within the UK contain a similar dsRNAs.

The profile of dsRNAs found in *A. bisporus* (V95, EU) *V. fungicola* (V7-3) and *T. harzianum* (T7, KPNT, T32, th1c) is not typical of either the Partitiviridae or Totiviridae families. The total dsRNA found in them may indicate the occurrence of multiple virus infections in these fungi. However, purified preparations of these viruses are often associated with dsRNA species of unknown origin and could be satellite or defective dsRNA in nature (Buck, 1986).

Accumulation of defective dsRNA and/or satellite dsRNA is a common feature of mycovirus infections, and the resulting complexity of dsRNA banding pattern has, in some instances, led to confusion in interpreting the nature and organization of the virus genomes. In these cases, molecular characterization of the virus in question is required to verify whether the essential genome is segmented, nonsegmented plus defective/satellite dsRNA or whether the complexity is the result of mixed infections with more than one virus.

Harmsen, *et al.*, (1989) reported that they analyzed the dsRNA composition of 14 individual mushrooms (strain Horst U1) of a flush affected by La France disease. Eight mushrooms with a diseased appearance (malformed : long stipes, small caps) contained the " disease-related" dsRNA pattern. In addition, two out of six healthy-looking mushrooms harboured the typical disease-associated dsRNA pattern with all 10 dsRNA occurring in amounts similar to those in the diseased mushrooms.

The other four healthy-looking mushrooms had the dsRNA composition typical of non-infected crops, i.e. they contained only L6 as a major band. This again shows that the relationship between the presence of the dsRNA and the phenotypic expression of the disease is complex. The presence of the dsRNAs does not appear necessarily to lead to malformed fruit bodies but it always leads to reduced crop yields.

In previous experiments Sonnenberg and Van Griensven (1991) have shown that dsRNAs and viral particles can be transferred from one commercial line to another by anastomosis. None of the wild strains they tested, however, had obtained any of the disease specific dsRNAs even after hyphal contact with a diseased commercial line Horst U1 for 3 weeks. The same experiment showed that transfer of dsRNA between the commercial line Horst U1 and Horst U3 had occurred in all samples examined. One reason for the failure of transferring dsRNA could be the incompatibility or absence of any anastomosis between the commercial line and the wild strains.

This seems, however, unlikely as homokaryons isolated from the 6 wild strains by protoplasting, were compatible with one or both homokaryons of the commercial line used as a donor of dsRNA. Construction of hybrids between infected commercial homokaryons and wild homokaryons will now be used to screen for resistance in wild strains. In addition, since Van der Lende, *et al.*, (1994) have shown that the 34 nm virus particles can be isolated intact, infection of protoplasts of wild strains will also be possible. Those experiments could also be used to prove that La France disease in the commercial white button mushroom is indeed a viral disease. These experiments will fulfill Koch's postulates and, in addition, will show which viral components are necessary to cause the symptoms of La France disease (Sonnenberg, *et al.*, 1995 a).

Melzer and Bidochka, (1998) reported a high incidence of dsRNA in natural populations of entomopathogenic fungi from southern Ontario. Analysis of 73 isolates of *Metarhizium anisopliae* and 12 isolates of *Beauveria bassiana* revealed that 35.3% contained dsRNA. The variation in the dsRNA banding patterns is an indication of both the number of dsRNA mycoviruses present in natural populations, and the ability of these fungi to bear multiple infections. Banding patterns which appear as

combinations of other patterns support the multiple infection hypothesis. Leal, *et al.*, (1994) and Bogo, *et al.*,(1996) found dsRNA banding patterns in *M. anisopliae* which were generally 6-13 segments, ranging from 0.8-4.1 kb. The dsRNA segments found in isolates of their study, however, were generally fewer in number but larger in size. Some of the dsRNA infections found in their study resemble the *Partitiviridae* families of dsRNA mycoviruses, which are associated with latent infections. *Partitiviridae* members have two dsRNA segments ranging from 1.4-3.0 kb (Murphy *et al.*, 1995).

Several studies have suggested that latent viral infections may be used as epidemiological markers for pathogenic fungi (Howitt, *et al.*, 1995 ; Newhouse, *et al.*, 1992), including *M. anisopliae* (Leal, *et al.*, 1994). In studies by Melzer and Bidochka (1998), dsRNA banding patterns were either distinctive for an isolate or could be shared amongst *M. anisopliae* isolates from southern Ontario. They demonstrated that dsRNA banding patterns are not necessarily geographically distinctive nor are they species specific. Their results suggest that dsRNA banding patterns are not recommended for the epidemiological studies with entomopathogenic fungi.

In this study, purification of virus-like particles (VLPs) from fruit bodies and fungal mycelia was successful and preparation obtained were suitable for further studies including electron microscopy and determination of the protein composition of the VLPs. Critical steps in the purification process of fungi could be noted as follows: (1) all of the steps needed to been done at 4°C. (2) Clarification with chloroform for longer than 45 min would damage the virus particles. (3) In the PEG precipitation step, 0.5M NaCl seemed necessary to limit irreversible aggregation of virus in PEG (10%) pellets. Slow resuspension of virus in the PEG pellets by gentle stirring in the cold room overnight appeared to give higher yields than a rapid suspending method.

Van Zaayen and Igesz, (1969) studied the cytology of virus-diseased mycelial cells of *A. bisporus*, and found virus particles in aggregates, often near a septum or close to the nucleus. The aggregates were partly surrounded by membranes of the

endoplasmic reticulum system. Most probably viroplasms were observed. Only one type of virus particle has been found, with a diameter of approximately 25 nm. Some particles had a dark centre, as was also noticed in the pelleted virus.

Liang, *et al.*, (1990) reported that in south China a high concentration of virus can be extracted from both deformed (dwarfed or elongated stipes) or apparently healthy fruit bodies of *Pleurotus ostreatus*, but in north China, virus particles are hardly found. This condition as the same as the report by Ushiyama (1979), that no VLPs were found in other cultivated mushrooms in Japan including *Pholiota nameko* and *Pleurotus ostreatus*. It seems that the presence of VLPs varies in different areas.

Two totiviruses were found to coinfect the filamentous fungus *Sphaeropsis sapinea*, a well known pathogen of pines. Isometric VLPs approximately 35 nm in diameter were isolated from extracts of this fungus. The linear genomes of 5163 bp (SsRV1) and 5202 bp (SsRV2), respectively, were identically organized with two large overlapping ORFs. The mode of translation and the genomic organization are similar to those of Helminthosporium victoriae 190S virus. Hv190SV thus appears to be closely related to the SsRVs. Interestingly, based on amino acid sequence homology SsRV1 was more closely related to Hv190SV than to SsRV2.(Preisig, *et al.*, 1998). Moreover, mixed viral infections have been demonstrated in *Aspergillus* and *Penicillium* (Benigni, *et al.*, 1977 ; Buck and Ratti, 1977).

The relationship of growth rate , morphological changes, conidiation to the degree of virulence was also examined and compared with isogenic isolates. These results provide evidence for the effect of dsRNA in the biological processes of these fungi. A targeted disruption of G-protein α subunit gene was found in the chestnut blight fungus *C. parasitica* infected with dsRNA. These revealed roles for the $G_{i\alpha}$ subunit CPG-1 in fungal reproduction, virulence and vegetative growth (Kasahara and Nuss, 1997).

In the case of the chestnut blight fungus, it has been demonstrated that the virus-mediated changes do not result from a general debilitation of the host, but rather from the differential regulation of specific genes. Thus, ORF A of the virus was identified as the determinant of traits associated with alterations of fungal phenotype, such as reduced pigmentation, and its proteolytic processing was, for instance, demonstrated to be required for reduction of fungal conidiation. ORF B controls virulence attenuation, and recent progress in the elucidation of the signal transduction pathways that govern fungal pathogenesis has identified the role of G protein-mediated cAMP accumulation on the reduction of fungal virulence (Nuss, 1996).

Choi, *et al.*, (1992) report evidence for hypovirulence-associated viruses (HAV)-mediated modulation of the expression of a specific, well characterized fungal gene encoding a known protein product. Of equal importance, they were able to demonstrate that the expression of three additional fungal genes, those encoding β-tubulin, actin, and glyceraldehyde-3-phosphate dehydrogenase, was not significantly altered in hypovirulent strains under the same conditions. These results support the proposal that the phenotype is a consequence of the altered expression of a specific subset of fungal genes rather than a general debilitation of the fungus. Moreover, a report that hypovirulence-associated characteristics can be conferred by transformation of a virulent, virus-free strain with a cDNA copy of a portion of the HAV dsRNA (Choi and Nuss, 1992) suggests that the altered expression of fungal genes is due to the action of specific viral gene products rather than a general response to the physical presence of the viral RNA.

The present results demonstrate that rapid cloning of the dsRNA gene can be achieved directly from a small amount of total dsRNA using RT-PCR. The current procedure makes it possible to omit the many complicated and time-consuming steps. The present RT-PCR procedure could also be applied to rapid cDNA cloning of other dsRNA viruses if the nucleotide sequence of both terminal portion of expected gene is available. In the case of cDNA clones of dsRNA of *V. fungicola* isolate V7-3 and *T. harzianum* isolate T7, they are new dsRNA-viruses where published sequences are not available. Partial nucleotide sequence was derived from use a random primere

RT-PCR protocol. Further work should focus on completing the sequences by using 5′ RACE (Rapid Amplification of cDNA Ends) which will allow the amplification of unknown sequences at the 5′end of the RNA.

These studies present the first report indicating partial amino acid sequence similarity dsRNA-3 of *T. harzianum* T7 with the fungal guanine nucleotide-binding protein alpha-3 subunit (G-protein) of *Ustilago maydis*. This might be the cause of down-regulation of G-protein production in the fungal host. Mechanistically, this could be analogous to the reduction of gene expression. An alternative mechanism might involve interference with signal transduction pathways, direct interaction of dsRNA-encoded polypeptides with repressor molecules or stabilization of mRNA turnover must also be considered. The down-regulation of specific fungal polypeptides by the dsRNAs that cause hypovirulence of *C. parasitica* (Powell and Van Alfen, 1987) suggests that the viruses are affecting normal regulatory functions of their fungal host. The characterization of this viral-regulated polypeptide is an important step in these studies of how fungal gene expression is affected by the viruses responsible for hypovirulence. In this regard, recent results several evidences have provided new opportunities for examining the molecular basis of hypovirulence (Nuss, 1996).

Future experiments should aim to provide further sequence data for *A. bisporus*, *V. fungicola* and *T. harzianum* with a view to understanding the role and function of encoded protein products. Studies on the expression of viral genes via internal initiation mechanisms are presently attracting considerable general interest. Because dsRNAs in *A. bisporus*, *V. fungicola* and *T. harzianum* have been shown to effect biological functions in fungal host. Further studies of genome structure and expression strategy will be valuable for understanding the role in pathogenesis and the potential use as a biocontrol agent.

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APPENDICES

APPENDICES

Appendix I: Buffer and Media

LB (Luria-Bertani) Medium

Per litre:

Bacto-tryptone	10	g
Bacto-yeast extract	5	g
NaCl	10	g

Adjust pH to 7.4 with NaOH.

SOC Medium

Per 100ml :

Bacto-tryptone	2.0	g
Bacto-yeast extract	0.5	g
1 M NaCl	1	ml
1 M KCl	0.25	ml
2 M Mg^{2+}stock	1	ml

(1M $MgCl_2$.$6H_2O$/ 1M $MgSO_2$.$7H_2O$), filter-sterilized

2 M glucose	1	ml

(filter-sterilized, 0.2 μm filter unit)

The final pH should be 7.0

Denhardt's solution 50X

Ficoll	5	g
bovine serum albumin (BSA)	5	g
polyvinylpyrrolidone (PVP)	5	g
H_2O	500	ml

Sterile filter. Dispense into 25 ml aliquots and store at -20°C.

PIPES buffer (0.3 M)

Per 100 ml:

Piperazine-N, N-bis (2-ethanesulfonic acid)	9	g

0.1 M sodium hydroxide

Add 9.0 g of PIPES to 50 ml water, and then add 0.1 m NaOH, while stirring, until the powder dissolves. The final pH should be 6.8 and then make up to 100 ml with H_2O.

SSC buffer 20X

Per litre:

NaCl (3M)	175.3 g
Na_3 Citrate.$2H_2O$ (0.3 M)	88.2 g
Add H_2O	800 ml

Add 10 N NaOH to pH to 7.0

Add H_2O to make 1 liter

Sterilize by autoclaving ; all components will go into solution in autoclave.

SSPE buffer 20X

Per litre:

NaCl (3M)		175.3 g
$NaH_2PO_4 . H_2O$ (0.2 M)		27.6 g
EDTA-Na_2	(0.2 M)	40 ml of 0.5 EDTA stock solution
Add H_2O		800 ml

Add 10 N NaOH to pH to 7.4

Add H_2O to make 1 liter

Sterilize by autoclaving ; all components will go into solution in autoclave.

Tris-Borate (TBE)

Working solution :

0.08 M. Tris-phosphate

0.008 M. EDTA

Stock solution 5x :

Per litre :

Tris base	54	g
boric acid	27.5	g
EDTA (0.05 M , pH 8.0)	20	ml
H_2O to make	1	liter

TE buffer (Tris/EDTA)

pH 8.0

10 mM Tris.Cl (pH 8.0)

1 mM EDTA (pH 8.0)

STE buffer

Working solution :

50 mM Tris-base

100 mM NaCl

1 mM $NA_2EDTA .H_2O$

Stock solution 10x :

Per litre:

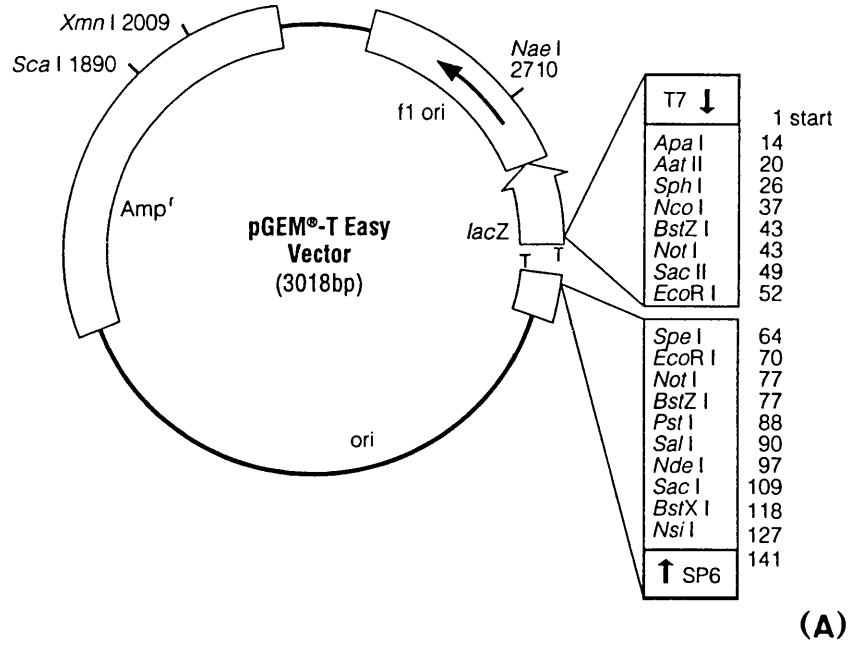
Tris-base	61	g
NaCl	58	g
$NA_2EDTA .H_2O$	3.7	g

Add Conc. HCl to pH 6.8

Add H_2O to make 1 liter

Sterilize by autoclaving ; all components will go into solution in autoclave.

Appendix I: Vectors



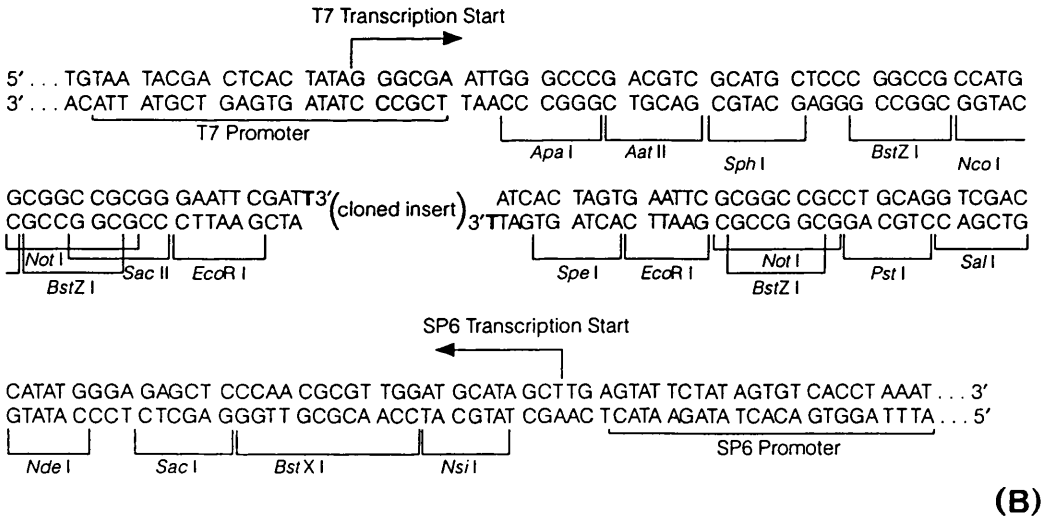


Fig. I A : pGEM®-T Easy Vector circle maps.

B : The promotor and multiple cloning sequence of pGEM® -T Easy vector. The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA Polymerase.

Appendix II

Appendix II-1

Two Sample T-Test and Confidence Interval

Comparison of dry weight (mg)

II-1.1

Two sample test for *A. bisporus* dsRNA-free VS *A. bisporus* +dsRNA isolateV95

	No. replication	Mean	StDev	SE Mean
dry weight (dsRNA-free)	10	42.90	5.61	1.8
dry weight (+dsRNA ,V95)	10	21.60	8.61	2.7

95% CI for mu dsRNA-free - mu +dsRNA V95 : (14.3, 28.3)

T-Test mu dry weight (dsRNA-free) ≠ dry weight (+dsRNA ,V95) :

T = 6.53 P = 0.0000 DF = 15

II-1.2

Two sample test for *A. bisporus* dsRNA-free VS *A. bisporus* +dsRNA isolate EU

	No. replication	Mean	StDev	SE Mean
dry weight (dsRNA-free)	10	42.90	5.61	1.8
dry weight (+dsRNA ,EU)	10	13.00	6.65	2.1

95% CI for mu dsRNA-free - mu +dsRNA EU : (24.1, 35.7)

T-Test mu dry weight (dsRNA-free) ≠ dry weight (+dsRNA ,EU) :

T = 10.87 P = 0.0000 DF = 17

Appendix II-2

Two Sample T-Test and Confidence Interval

Comparison of dry weight (mg)

II-2.1

Two sample test for *V. fungicola* V7-3h heat treated VS *V. fungicola* V7-3 +dsRNA

	No. replication	Mean	StDev	SE Mean
dry weight (V7-3h heat treated)	10	124.30	8.22	2.6
dry weight (V7-3 +dsRNA)	10	96.2	11.6	3.7

95% CI for mu V7-3h heat treated - mu V7-3 +dsRNA : (18.6, 37.6)

T-Test mu dry weight (heat treated) ≠ dry weight (+dsRNA) :

T = 6.26 P = 0.0000 DF = 16

II-2.2

Two sample test for *T. harzianum* T7h heat treated VS *T. harzianum* T7 +dsRNA

	No. replication	Mean	StDev	SE Mean
dry weight (T7 heat treated)	10	8.360	0.430	0.14
dry weight (T7 +dsRNA)	10	5.090	0.547	0.17

95% CI for mu T7h heat treated - mu T7 +dsRNA : (2.81, 3.73)

T-Test mu dry weight (heat treated) ≠ dry weight (+dsRNA) :

T = 14.87 P = 0.0000 DF = 17

Appendix II-3

Two Sample T-Test and Confidence Interval

Comparison of spore production

II-3.1

Two sample test for *V. fungicola* V7-3h heat treated VS *V. fungicola* V7-3 +dsRNA

	No. replication	Mean	StDev	SE Mean
spore production (V7-3h heat treated)	10	40.60	6.41	2.0
spore production (V7-3 +dsRNA)	10	23.00	10.30	3.3

95% CI for mu V7-3h heat treated - mu V7-3 +dsRNA : (9.5, 25.8)

T-Test mu spore production (heat treated) ≠ spore production (+dsRNA) :

T = 4.59 P = 0.0004 DF = 15

II-3.2

Two sample test for *T. harzianum* T7h heat treated VS *T. harzianum* T7 +dsRNA

	No. replication	Mean	StDev	SE Mean
spore production (T7 heat treated)	10	17.53	3.88	1.20
spore production (T7 +dsRNA)	10	10.61	1.22	0.39

95% CI for mu T7h heat treated - mu T7 +dsRNA : (4.1, 9.79)

T-Test mu spore production (heat treated) ≠ spore production (+dsRNA) :

T = 5.38 P = 0.0003 DF = 10

Appendix II-4

Two Sample T-Test and Confidence Interval

Comparison of lesion radius (mm)

II-4.1

Two sample test for *V. fungicola* V7-3h heat treated VS *V. fungicola* V7-3 +dsRNA

	No. replication	Mean	StDev	SE Mean
leasion radius (V7-3h heat treated)	15	18.33	0.673	0.17
leasion radius (V7-3 +dsRNA)	15	12.67	0.417	0.11

95% CI for mu V7-3h heat treated - mu V7-3 +dsRNA : (0.14, 0.99)

T-Test mu leasion radius (heat treated) ≠ leasion radius (+dsRNA) :

T = 2.77 P = 0.011 DF = 23

II-4.2

Two sample test for *T. harzianum* T7h heat treated VS *T. harzianum* T7 +dsRNA

	No. replication	Mean	StDev	SE Mean
lesion radius (T7 heat treated)	15	24.20	0.353	0.091
lesion radius (T7 +dsRNA)	15	22.47	0.250	0.065

95% CI for mu T7h heat treated - mu T7 +dsRNA : (-0.057, 0.403)

T-Test mu lesion radius (heat treated) ≠ lesion radius (+dsRNA) :

T = 1.55 P = 0.13 DF = 25

Appendix II-5

Two Sample T-Test and Confidence Interval

Comparison of disease progress on mushroom

II-5.1

Two sample test for *V. fungicola* V7-3h heat treated VS *V. fungicola* V7-3 +dsRNA

	No. replication	Mean	StDev	SE Mean
disease progress (V7-3h heat treated)	15	4.533	0.743	0.19
disease progress (V7-3 +dsRNA)	15	1.800	0.414	0.11

95% CI for mu V7-3h heat treated - mu V7-3 +dsRNA : (2.28, 3.19)

T-Test mu disease progress (heat treated) ≠ disease progress (+dsRNA) :

T = 12.44 P = 0.0000 DF = 21

II-5.2

Two sample test for *T. harzianum* T7h heat treated VS *T. harzianum* T7 +dsRNA

	No. replication	Mean	StDev	SE Mean
disease progress (T7 heat treated)	15	2.400	0.507	0.13
disease progress (T7 +dsRNA)	15	1.733	0.458	0.12

95% CI for mu T7h heat treated - mu T7 +dsRNA : (0.30, 1.03)

T-Test mu disease progress (heat treated) ≠ disease progress (+dsRNA) :

T = 3.78 P = 0.0008 DF = 27

Appendix III

Appendix III-1

emb|X94361.1|ABV1L1 Agaricus bisporus virus 1 (ABV1) L1 dsRNA
Length = 3396

Score = 5390 bits (2719), Expect = 0.0
Identities = 3245/3404 (95%), Gaps = 21/3404 (0%)
Strand = Plus / Plus

```
V95uk: 1    acttgtttgaatatgttgagtggatatgatagagaccagataattgggtgggctgaggat 60
            ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
ABV1 : 1    acttgtttgaatatgttgagtggatatgatagtgaccagataattgggtgggctgaggat 60

V95uk: 61   attccaaattttgatgttgagtacgcttgcatttcacacacacgtgcgaaagcagtggtt 120
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
ABV1 : 61   attccaaattttgatgttgagtatgcttgcatttcacacacacgtgcgaaagcagtggtt 120

V95uk: 121  ggtgaggatattgctcataatttgtcctgctattcaaggttacgtaccgcattgtctaat 180
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
ABV1 : 121  ggtgaggacattgctcataatttgtcctgctattcaaggttacgtaccgcattgtctaat 180

V95uk: 181  ataaatccagacgataatgcgcctttcatttttgtccagacttgtgaactggcagaagca 240
            ||||| |||||||||||||||||||||||||||||||| |||||||||| |||||||||
ABV1 : 181  ataaacccagacgataatgcgcctttcatttttgtccaaacttgtgaactagcagaagca 240

V95uk: 241  attggagtacagattgcgatggtgttgatagtcaaggaggggataattcaacaaactgat 300
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
ABV1 : 241  attggagtacagattgcgatggtattgatagtcaaggaggggataattcaacaaactgat 300

V95uk: 301  aggtataaaaatttgtcaagagaagaacagaaaactcttaagcatgtgcatgatgttcaa 360
            |||||||||| |||||||||||||||||||||||||||||| ||||||||||||||||||
ABV1 : 301  aggtataaaagtttgtcaagagaagaacagaaaactcttaaacatgtgcatgatgttcaa 360

V95uk: 361  aataatcataataaattgtgggttgaaaaatataagtatacgcatagggtcgtcttatct 420
            |||||||||||||||||||||| |||||||||||||||||||||||||| |||||||||
ABV1 : 361  aataatcataataaattgtgggtagaaaaatataagtatacgcatagggttgtcttatct 420

V95uk: 421  tataatgatatttaccacaaggttaagggattatatattagtgttgggtgtgtgaaaagc 480
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
ABV1 : 421  tataatgatatttaccacaaggttaagggattatatattagtgttgggtgtgtcaaaagc 480

V95uk: 481  tcctttggcacagatgatgtgttgagtgggatagatgagtatacttacgatgctcgcgag 540
            || ||||| ||||| |||||| |||||||||||||||||| ||||| ||||||||||||
ABV1 : 481  tcttttggtacagacgatgtgctgagtgggatagatgagtacacttatgatgctcgcgag 540

V95uk: 541  aaggtgaatgaggtttcgaatttgcttaaagagtcgaagcatccggcgaccaaagctgta 600
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABV1 : 541  aaggtaaatgaggtttcgaatttgcttaaagagtcgaagcatccggcgaccaaagctgta 600

V95uk: 601  tgcgcgaggtatttgcagcaaaattttcaggcggcagcgccagattatgctaatgcaagt 660
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
ABV1 : 601  tgcgcgaggtatttgcagcaaaattttcaggcggcagcgccagactatgctaatgcaagt 660

V95uk: 661  tgttcccacgttgcgtggtcgagatgggtaaatgcagtcaaatcgcacgttagaactgaa 720
            || |||||||||||||||||||||||| |||||||||||||||||| || ||||||
ABV1 : 661  tgctcccacgttgcgtggtcgagatgggtgaatgcagtcaaatcgcacgtcaggactgaa 720

V95uk: 721  agtatgcattatccaacaaacaatatggtaatggccatgtgcgacgaagagtatgtgata 780
            ||   |||||||||||||| |||||||| |||||||||| ||||| ||||||||||||
ABV1 : 721  agcgcgcattatccaacaaataatatggtgatggccatgtgtgacgaggagtatgtgata 780
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V95uk: 781  gagaactttcctcatcaagcagggtcagacaagtttgctgcctgtcatataagtacagtg 840
            ||||| ||||||||||||||||||||||| |||||||||||||||||||||||||||||
ABV1 : 781  gagaattttcctcatcaagcagggtcagataagtttgctgcctgtcatataagtacagtg 840

V95uk: 841  atcaaggatcacatggataagttgaaaaaaccaacaactggcagacctgtgtttgttcag 900
            |||||||||||||||||||||||||||||||| | ||| ||||||||||||||||||
ABV1 : 841  atcaaggatcacatggataagttgaaaaaaccaacgaatggtagacctgtgtttgttcag 900

V95uk: 901  actgcgtgtttgattggttcggtcttgaagaacaaagatatccagtgttcgtatgagaag 960
            ||||| |||||||||||||||||||||||| |||||||||||||| ||||||||||||
ABV1 : 901  actgcatgtttgattggttcggtcttgaagaataaagatatccagtgctcgtatgagaag 960

V95uk: 961  ctattgtcttttatattatatgaaatgttcttgtacacaacggatgttgagatgcataaa 1020
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABV1 : 961  ctattgtcttttatattatatgaaatgttcttgtacacaacggatgttgagatgcataaa 1020

V95uk: 1021 ttgtggagcagtgtggtgtattcgcctgtagataaagatactttctttttgtgtgcagag 1080
            |||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||
ABV1 : 1021 ttgtggagtagtgtggtatattcgcctgtagataaagatactttctttttgtgtgcagag 1080

V95uk: 1081 tatgctcatgcgaggataaggacatcgggaacgtatttaggtataaaattgactg--cg- 1137
            |||||||||||||||||||||||| |||||||||||||| ||||||||||||  ||
ABV1 : 1081 tatgctcatgcgaggataaggacatcaggaacgtatttaggcataaaattgactggccgt 1140

V95uk: 1138 -caacagatgtcatatatgttgtatccgcaatgtttag-ctggtagggtgtatggcaaag 1195
             |||||||||||||||||||||||||||||||||||| ||||||| ||||||||||||
ABV1 : 1141 ccaacagatgtcatatatgttgtatccgcaatgtttagcctggtagagtgtatggcaaag 1200

V95uk: 1196 aggatttggcacaagagtttgacgatcgtacttctgataggccggcgaagaggttctggt 1255
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABV1 : 1201 aggatttggcacaagagtttgacgatcgtacttctgataggccggcgaagaggttctggt 1260

V95uk: 1256 ccaatggagtgc-tgagcgaagaagaatataacataaggtttaataatgcaatactgaac 1314
            | |||||||||| | |||||||||||||||| |||||||| |||||||| |||||||||
ABV1 : 1261 ctaatggagtgcttaagcgaagaagaatataatataaggttcaataatgcgatactgaac 1320

V95uk: 1315 tgttattcgcatatgacaaccggatatagtgctaaatttaggcggttggcgaatgatgtt 1374
            |||||||||||||||||| |||||||||||||| ||| ||||||||||||||||||||
ABV1 : 1321 tgttattcgcatatgacaactggatatagtgctaagtttgggcggttggcgaatgatgtt 1380

V95uk: 1375 gaatcgttcaaatcgtttatgcagttacgaaaacgctggtcacagccgggtt--cggcaa 1432
            |||||||||||||||||||||||| ||||| | |||||||||||| ||||  ||||||
ABV1 : 1381 gaatcgttcaaatcgtttatgcagttgcgaaagc-ctggtcacagccaggttcccggcaa 1439

V95uk: 1433 ctggtgcaccgaaagttattcttagattgacggaacatgtggctgagttagatgccgatg 1492
            | |||||| |||||||||||| |||||||||||||||| ||||||| |||||||| |||
ABV1 : 1440 ccggtgca-cgaaagttattattagattgacggaacatatggctgaattagatgctgat- 1497

V95uk: 1493 cgcttcagctgggtgagttgttgactgatgccatcgataggattcaattgagacttaaca 1552
              |||||||| |||||||| ||||||||| |||||||||||||||||||||||||||||
ABV1 : 1498 -ccttcagct-ggtgagttattgactgatcccatcgataggattcaattgagacttaaca 1555

V95uk: 1553 agtcaacattatttgaattttctg-ccgtagtagaagcggtggaggctgcctggcggaac 1611
            |||||||||||  |||||||||||| |||||||||||||||||| ||||||||||||||
ABV1 : 1556 agtcaacatt-gttgaattttctgcccgtagtagaagcggtggaagctgcctggcggaat 1614

V95uk: 1612 tatgatccgaatagttttactggtgtgttctggaaacatgaagtgggaaaaaacgcatct 1671
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ABV1 : 1615 tatgatccgaatagttttactggtgtgttctggaaacatgaagtagggaaaaacgcatct 1674

V95uk: 1672 cggtcactgtggccagcgcatctagtccattatgttctagtgtccatgatattacactta 1731
            || ||| |||||||||||||||||||||||| |||||||||||||||||| | ||||||
ABV1 : 1675 cgatcattgtggccagcgcatctagtccattacgttctagtgtccatgatactgcactta 1734

V95uk: 1732 atcgacaagtcaggggaaataccaggatcacgtaacaatgcacctagtgataggcaattg 1791
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
ABV1 : 1735 atcgacaagtcaggagaaataccaggatcacgtaacaatgcacctagtgataggcaattg 1794
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V95uk: 1792 aaagatcactggatgtggtcagaatgccaagattttgtacctttaatgatggattacgct 1851
           || ||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
ABV1 : 1795 aaggatcactggatgtggtcagaatgccaagattttgtgcctttaatgatggattacgct 1854

V95uk: 1852 aattttaacgaacagcatagcatagaggcgatgaaagcaacaatacagccactgcgcgat 1911
           |||||||| |||||||||||||||||||||||||||||||||| ||||| ||||||||||||
ABV1 : 1855 aattttaatgaacagcatagcatagaggcgatgaaagcaacgatacaaccactgcgcgat 1914

V95uk: 1912 gtatatgctaaggcgggagtgctgtctaaagatctagctgatgctattgattgggtagtc 1971
           || |||||||||||| |||||| ||||||| ||||||||||||||||||||||||||||||
ABV1 : 1915 gtgtatgctaaggcaggagtgttgtctaaggatctagctgatgctattgattgggtagtc 1974

V95uk: 1972 aaatcgtttgatcaaatatgcgctatagatgaaaatggtgacttgaggcgatttacacac 2031
           || |||||||||||||||||||||||| ||||||||||||||||||||||||||||||
ABV1 : 1975 aagtcgtttgatcaaatatgcgctatagacgaaaatggtgacttgaggcgatttacacac 2034

V95uk: 2032 ggcttgttatctggatggagatgtacggcttacatcaataatttaataaatatagctcag 2091
           ||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABV1 : 2035 ggcctgctatctggatggagatgtacggcttacatcaataatttaataaatatagctcag 2094

V95uk: 2092 tacgaagtgggtagacaacagctgcatgagtttttggaatgagttcttcataccagttt 2151
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABV1 : 2095 tacgaagtgggtagacaacagctgcatgagtttttggaatgagttcttcataccagttc 2154

V95uk: 2152 gacacaggggagacgatggatgtgcagatgaaaaagacttggtgacagcttattcgttg 2211
           |||||||| ||||| |||||||||||||||| ||||||||||||||||||||||||||||
ABV1 : 2155 gacacaggaggagatgatggatgtgcagatgagaaagacttggtgacagcttattcgttg 2214

V95uk: 2212 ttgcgtagcatgactgctatgggatttgagtttaaggatataaaacaattaataagtagc 2271
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
ABV1 : 2215 ttgcgtagcatgactgctatgggatttgagtttaaggatataaaacaattaataagcagc 2274

V95uk: 2272 ggaacgcacgaatttttcagattgctaataactccagaaggtgtattcggttcagtgatc 2331
           |||||||||||||||| |||||| ||||||||||||||||||||||||||||||||||||
ABV1 : 2275 ggaacgcacgaatttttagattgttaataactccagaaggtgtattcggttcagtgatc 2334

V95uk: 2332 aggatgttagcatctgctgcatcaggccaatggtctaattcagtgagagcaaaattagtg 2391
           || |||||||| |||||||||||||||||||||| |||||||||||||| |||||||||
ABV1 : 2335 agaatgttagcgtctgctgcatcaggccaatggtccaattcagtgagagcgaaattagtg 2394

V95uk: 2392 gacccactttcaaagatgactagtataatggatataaaacataaattatggaggagatca 2451
           || |||||||||||||||||||||||||||| |||||||||||||||||||||||||||
ABV1 : 2395 gatccactttcaaagatgactagtataatggacataaaacataaattatggaggagatcg 2454

V95uk: 2452 ggttataatgatgattgggctgaacaattgcaacattatatgagcttgaaatgggtagat 2511
           |||||| |||||||||||||||||||||||||||||||||||||||| ||||||||||||||
ABV1 : 2455 ggttacaatgatgattgggctgaacaattgcaacattatatgagcctgaaatgggtagat 2514

V95uk: 2512 ccggagagcccaaaagatttgataaattttgtgcatggcacgaaaagtacaggagg---t 2568
           |||||||||||||| ||||||| |||||||||||||||  |||||| || |||||   |
ABV1 : 2515 ccggagagcccaaaggatttgacaaattttgtgcatggccagaaaagcacgggaggtttt 2574

V95uk: 2569 ttgggtataccagactgtcaaggtaggttatatgagttggcaagtgtgagtggaatagaa 2628
           ||||| |||||||||||||||||||| |||||||||| || ||||||||||||||||||
ABV1 : 2575 ttgggcataccagactgtcaaggtaggctatatgagttagcgagtgtgagtggaatagaa 2634

V95uk: 2629 aaaccatcaaaaatagaactgcgttcattcccgcacgatgcgagtgagcagttgctgaat 2688
           || |||||||||||||| |||| ||||||||||||||||||||||||||||||||||||
ABV1 : 2635 aagccatcaaaaatagaattgcgctcattcccgcacgatgcgagtgagcagttgctgaat 2694

V95uk: 2689 aaattagctccagacatagaacagatggtcggtgctgatcaaatggaagatatggaacgt 2748
           |||||| ||||||||||||||||||||||||||||||||||| ||||||||||||||||||
ABV1 : 2695 aaattggctccagacatagaacagatggtcggtgctgatcagatggaagatatggaacgt 2754

V95uk: 2749 atagctgtgaacatgagtaaaatggtgtttgttggcaacgtagcttcaggatttagtcca 2808
           |||||||||||||||||||||||||| |||||||| ||||||||||||||||||||||||
ABV1 : 2755 atagctgtgaacatgagtaaaatggtatttgttggtaacgtagcttcaggatttagtccg 2814
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V95uk: 2809 gctatagcaagtaaaatggtagaatctacaaatttgagaaaggtacgagcaagaaggaca 2868
           ||||||||||||||||||||||||||||||||||||||||||| |||||||||| |||||||
ABV1 : 2815 gctatagcaagtaaaatggtagaatctacaaatttgagaaaagtacgagcaaaaaggaca 2874

V95uk: 2869 ggcagggtatttgccgttgataatacatcgccttttaacgatagatgatgttaggaaagat 2928
           |||||||||||||| || ||||||||||||||||||||||||||||||||||||||||||||
ABV1 : 2875 ggcagggtatttgctgtcgataatacatcgccttttaacgatagatgatgttaggaaagat 2934

V95uk: 2929 cttgatttatggcaccctacgattgaagatattaagaaggtaattggtgactatagttcg 2988
           |||||||||||| |||||||||||||||||| ||||||||||||||||||||||||||||
ABV1 : 2935 cttgatttatggtaccctacgattgaagatatcaagaaggtaattggtgactatagttcg 2994

V95uk: 2989 atgagtcttttgactaaaccaacaagtcatgaaactttattgatgaagctaagcgtcaaa 3048
           |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
ABV1 : 2995 atgagtcttttgactaaaccaacaagtcatgaaactttattgatgaagttaagcgtcaaa 3054

V95uk: 3049 cataatgtaaaattatacagtaggttacattatatgttgatggctgagccggacataaca 3108
           |||||||||||||||||||||||||||||||||||||||||||||||| || |||||||||
ABV1 : 3055 cataatgtaaaattatacagtaggttacattatatgttgatggctgaaccagacataaca 3114

V95uk: 3109 ggtctgggaccaatattggcaactgaagattattacaaggatcttttgatattatctttt 3168
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
ABV1 : 3115 ggtctgggaccaatattggcaactgaagattattacaaggatcttttgatattgtctttt 3174

V95uk: 3169 ctgaccgcagaaaaactgaatgctagaggagtaagtcagaagttctcagattatgcggta 3228
           ||||| |||||||||||||||||||||||||||||||| |||||||||||||||| |||
ABV1 : 3175 ctgactgcagaaaaactgaatgctagaggagtaagtcaaaagttctcagattatgcagta 3234

V95uk: 3229 gctatggcaatgggtaaaaggatgaattactaaagcgagcaat-acgaatggaacaacca 3287
           |  |||||||||||||||||||||||||||||||||| |||||| | ||||||||||||||
ABV1 : 3235 g--atggcaatgggtaaaaggatgaattactaaagcaagcaataaagaatggaacaacca 3292

V95uk: 3288 aacgtacgtagcgtgctggttgttggtggaatgatcgaatcatacgtagtgtttcgatcg 3347
           || |||||||||||||||||||||||| |||||||||||||||||||||||||||||||
ABV1 : 3293 aatgtacgtagcgtgctggttgttggtgaaatgatcgaatcatacgtagtgtttcgatcg 3352

V95uk: 3348 ttaaattgtttaagtgaacacgtgtagtgtgcaacttaaatggc 3391
           |||||||||||||||||||||||||||||||||||||||||||
ABV1 : 3353 ttaaattgtttaagtgaacacgtgtagtgtgcaacttaaatggc 3396
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Fig. III.1 The nucleotide sequence of 3391 nt of dsRNA L1 *A. bisporus* isolate V95 from The UK compared with dsRNA L1 *A. bisporus* (ABV1) from The Netherlands.

Appendix III-2

emb|CAA64144.1| (X94361)
RNA-dependent RNA polymerase [Agaricus bisporus virus 1]
Length = 1078

Score = 1993 bits (5107), Expect = 0.0
Identities = 1001/1072 (93%),Positives = 1012/1072(94%),Gaps = 0/1072(0%)
Frame = +1

```
V95uk: 13   MLSGYDRDQIIGWAEDIPNFDVEYACISHTRAKAVVGEDIAHNLSCYSRLRTALSNINPD 192
           MLSGYD DQIIGWAEDIPNFDVEYACISHTRAKAVVGEDIAHNLSCYSRLRTALSNINPD
ABV1 : 1    MLSGYDSDQIIGWAEDIPNFDVEYACISHTRAKAVVGEDIAHNLSCYSRLRTALSNINPD 60

V95uk: 193  DNAPFIFVQTCELAEAIGVQIAMVLIVKEGIIQQTDRYKNLSREEQKTLKHVHDVQNNHN 372
           DNAPFIFVQTCELAEAIGVQIAMVLIVKEGIIQQTDRYK+LSREEQKTLKHVHDVQNNHN
ABV1 : 61   DNAPFIFVQTCELAEAIGVQIAMVLIVKEGIIQQTDRYKSLSREEQKTLKHVHDVQNNHN 120

V95uk: 373  KLWVEKYKYTHRVVLSYNDIYHKVKGLYISVGCVKSSFGTDDVLSGIDEYTYDAREKVNE 552
           KLWVEKYKYTHRVVLSYNDIYHKVKGLYISVGCVKSSFGTDDVLSGIDEYTYDAREKVNE
ABV1 : 121  KLWVEKYKYTHRVVLSYNDIYHKVKGLYISVGCVKSSFGTDDVLSGIDEYTYDAREKVNE 180

V95uk: 553  VSNLLKESKHPATKAVCARYLQQNFQAAAPDYANASCSHVAWSRWVNAVKSHVRTESMHY 732
           VSNLLKESKHPATKAVCARYLQQNFQAAAPDYANASCSHVAWSRWVNAVKSHVRTES HY
ABV1 : 181  VSNLLKESKHPATKAVCARYLQQNFQAAAPDYANASCSHVAWSRWVNAVKSHVRTESAHY 240

V95uk: 733  PTNNMVMAMCDEEYVIENFPHQAGSDKFAACHISTVIKDHMDKLKKPTTGRPVFVQTACL 912
           PTNNMVMAMCDEEYVIENFPHQAGSDKFAACHISTVIKDHMDKLKKPT GRPVFVQTACL
ABV1 : 241  PTNNMVMAMCDEEYVIENFPHQAGSDKFAACHISTVIKDHMDKLKKPTNGRPVFVQTACL 300

V95uk: 913  IGSVLKNKDIQCSYEKLLSFILYEMFLYTTDVEMHKLWSSVVYSPVDKDTFFLCAEYAHA 1092
           IGSVLKNKDIQCSYEKLLSFILYEMFLYTTDVEMHKLWSSVVYSPVDKDTFFLCAEYAHA
ABV1 : 301  IGSVLKNKDIQCSYEKLLSFILYEMFLYTTDVEMHKLWSSVVYSPVDKDTFFLCAEYAHA 360
                                                  1
V95uk: 1093 RIRTSGTYLGIKLTAQQMSYML-------YPQCLAGRVYGKEDLAQEFDDRTSDRPAKRF 1251
           RIRTSGTYLGIKLT +    +          +C+A R++ K         R
ABV1 : 361  RIRTSGTYLGIKLTGRPTDVIYVVSAMFSLVECMAKRIWHKSLTIVLLIGRRRGSGLME- 419
                                                                  2
V95uk: 1252 WSNGVLSEEEYNIRFNNAILNCYSHMTTGYSAKFRRLANDVESFKSFMQLRKRWSQPGSA 1431
                LSEEEYNIRFNNAILNCYSHMTTGYSAKF RLANDVESFKSFMQLRK
ABV1 : 420  ----CLSEEEYNIRFNNAILNCYSHMTTGYSAKFGRLANDVESFKSFMQLRKPGHSQVPG 475

V95uk: 1432 TGAPKVILRLTEHVAELDADALQLGELLTDAIDRIQLRLNKSTLFEFSAVVEAVEAAWRN 1611
              KVI+RLTEH+AELDAD    GELLTD IDRIQLRLNKSTL  F  VVEAVEAAWRN
ABV1 : 476  NRCTKVIIRLTEHMAELDADP-SAGELLTDPIDRIQLRLNKSTLLNFLPVVEAVEAAWRN 534
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                                   3
V95uk: 1612 YDPNSFTGVFWKHEVGKNASRSLWPAHLVHYVLVSMILHLIDKSGEIPGSRNNAPSDRQL 1791
            YDPNSFTGVFWKHEVGKNASRSLWPAHLVHYVLVSMILHLIDKSGEIPGSRNNAPSDRQL
ABV1 : 535  YDPNSFTGVFWKHEVGKNASRSLWPAHLVHYVLVSMILHLIDKSGEIPGSRNNAPSDRQL 594
                                  4
V95uk: 1792 KDHWMWSECQDFVPLMMDYANFNEQHSIEAMKATIQPLRDVYAKAGVLSKDLADAIDWVV 1971
            KDHWMWSECQDFVPLMMDYANFNEQHSIEAMKATIQPLRDVYAKAGVLSKDLADAIDWVV
ABV1 : 595  KDHWMWSECQDFVPLMMDYANFNEQHSIEAMKATIQPLRDVYAKAGVLSKDLADAIDWVV 654
                                                5
V95uk: 1972 KSFDQICAIDENGDLRRFTHGLLSGWRCTAYINNLINIAQYEVGRQQLHEFFGMSSSYQF 2151
            KSFDQICAIDENGDLRRFTHGLLSGWRCTAYINNLINIAQYEVGRQQLHEFFGMSSSYQF
ABV1 : 655  KSFDQICAIDENGDLRRFTHGLLSGWRCTAYINNLINIAQYEVGRQQLHEFFGMSSSYQF 714
                6                                       7          8
V95uk: 2152 DTGGDDGCADEKDLVTAYSLLRSMTAMGFEFKDIKQLISSGTHEFFRLLITPEGVFGSVI 2331
            DTGGDDGCADEKDLVTAYSLLRSMTAMGFEFKDIKQLISSGTHEFFRLLITPEGVFGSVI
ABV1 : 715  DTGGDDGCADEKDLVTAYSLLRSMTAMGFEFKDIKQLISSGTHEFFRLLITPEGVFGSVI 774

V95uk: 2332 RMLASAASGQWSNSVRAKLVDPLSKMTSIMDIKHKLWRRSGYNDDWAEQLQHYMSLKWVD 2511
            RMLASAASGQWSNSVRAKLVDPLSKMTSIMDIKHKLWRRSGYNDDWAEQLQHYMSLKWVD
ABV1 : 775  RMLASAASGQWSNSVRAKLVDPLSKMTSIMDIKHKLWRRSGYNDDWAEQLQHYMSLKWVD 834

V95uk: 2512 PESPKDLINFVHGTKSTGG-LGIPDCQGRLYELASVSGIEKPSKIELRSFPHDASEQLLN 2688
            PESPKDL NFVHG KSTGG LGIPDCQGRLYELASVSGIEKPSKIELRSFPHDASEQLLN
ABV1 : 835  PESPKDLTNFVHGQKSTGGFLGIPDCQGRLYELASVSGIEKPSKIELRSFPHDASEQLLN 894

V95uk: 2689 KLAPDIEQMVGADQMEDMERIAVNMSKMVFVGNVASGFSPAIASKMVESTNLRKVRARRT 2868
            KLAPDIEQMVGADQMEDMERIAVNMSKMVFVGNVASGFSPAIASKMVESTNLRKVRA+RT
ABV1 : 895  KLAPDIEQMVGADQMEDMERIAVNMSKMVFVGNVASGFSPAIASKMVESTNLRKVRAKRT 954

V95uk: 2869 GRVFAVDNTSPLTIDDVRKDLDLWHPTIEDIKKVIGDYSSMSLLTKPTSHETLLMKLSVK 3048
            GRVFAVDNTSPLTIDDVRKDLDLW+PTIEDIKKVIGDYSSMSLLTKPTSHETLLMKLSVK
ABV1 : 955  GRVFAVDNTSPLTIDDVRKDLDLWYPTIEDIKKVIGDYSSMSLLTKPTSHETLLMKLSVK 1014

V95uk: 3049 HNVKLYSRLHYMLMAEPDITGLGPILATEDYYKDLLILSFLTAEKLNARGVSQKFSDYAV 3228
            HNVKLYSRLHYMLMAEPDITGLGPILATEDYYKDLLILSFLTAEKLNARGVSQKFSDYAV
ABV1 : 1015 HNVKLYSRLHYMLMAEPDITGLGPILATEDYYKDLLILSFLTAEKLNARGVSQKFSDYAV 1074
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Fig.III.2 The amino acid sequence of dsRNA L1 *A. bisporus* isolate V95 from The UK compared with dsRNA L1 *A. bisporus* (ABV1) from The Netherlands.

Appendix III-3

emb|X94362.1|ABV1L5 Agaricus bisporus virus 1 (ABV1) L5 dsRNA
Length = 2455

Score = 3493 bits (1762), Expect = 0.0
Identities = 2225/2371 (94%), Gaps = 1/2371 (0%)
Strand = Plus / Plus

```
V95uk: 1    ggataagtggttagtgcaagtagaattgattaggcaacggctagttggccaaattgaatc 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABV1 : 1    ggataagtggttagtgcaagtagaattgattaggcaacggctagttggccaaattgaatc 60

V95uk: 61   atcgaacaaaacctcaacgcaaatttcataacaacatttgaatttgttcgtgcttacttt 120
             ||||||||| ||| ||||||| |||||||| ||||||||||||||||||||||||||||
ABV1 : 61   gtcgaacaaa-ccttaacgcaattttcataataacatttgaatttgttcgtgcttacttt 119

V95uk: 121  aaaacatgactacacaacaagcaagtgtaatgcggttcacaactgcctattctagtaata 180
            ||| ||||||||||||||||||||||||||||||||||||||| ||||||||||||||
ABV1 : 120  aaaccatgactacacaacaagcaagtgtaatgcggttcacaactgcttattctagtaata 179

V95uk: 181  acaaagacgcgaatgacgtggtgcctgggagtctgttgcatcaaagattggtgaagagtc 240
            ||||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||
ABV1 : 180  acaaaaacgcgaatgatgtggtgcctgggagtctgttgcatcaaagattggtgaagagtc 239

V95uk: 241  gggagttagaagagcatgttacgcggaattatggtttgccagatgtgatgtactcatatg 300
            | ||||| |||||||||||||||||||||||||| ||||||||||||||||||| ||||
ABV1 : 240  gagagttggaagagcatgttacgcggaattatggtctgccagatgtgatgtactcgtatg 299

V95uk: 301  ctggcaccttacctggtgaatacgcgaggattgggagtcaaattgagaaacagataatag 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
ABV1 : 300  ctggcaccttacctggtgaatacgcgaggattgggagtcaaattgagaaacaaataatag 359

V95uk: 361  ggaagaatctatccaatatactgctggagttatgtgatggagataatatggaacgggtga 420
            |||| |||||||| || |||||||| |||||||||||||||| |||||||||| ||||
ABV1 : 360  ggaaaaatctatctaacatactgctagagttatgtgatggagacaatatggaacgagtga 419

V95uk: 421  agatggtgactttatgccatgatgtaatgcataataaagtgcagaagatagcagcaagtc 480
            ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
ABV1 : 420  agatggtgactttatgccatgatgtaatgcataataaagtgcagaaaatagcagcaagtc 479

V95uk: 481  gcacttcgtgtaagttcgaagtaagtgagcagagtgatatggaggttaaaattgaagtgg 540
            | |||||||||||| |||||||||||||||| || || ||||||||||||||||||||||
ABV1 : 480  gtacttcgtgtaaattcgaagtaagtgagcaaagcgacatggaggttaaaattgaagtgg 539

V95uk: 541  cgttcggatcggccgaagatggctaccaaacaaaggaattggtgattacttttgaaaagg 600
            ||||||| ||| |||||||||||  |||||||| |||||||||| |||||||||| |
ABV1 : 540  cgttcgggtcgcccgaagatggctgtcaaacaaaagaattggtgataacttttgaaaaag 599

V95uk: 601  taaatgatcagtgggcgtttgtaggtatggaggcattgaatgattttagaaaggtcagaa 660
            | |||||||||||||||| ||||||||||||||||||||||||||||||||| ||||
ABV1 : 600  tgaatgatcagtgggcgttcgtaggtatggaggcattgaatgattttagaaaggttagaa 659

V95uk: 661  atatgtacagagcgcgtgcagcaaaaaaacaattgcaggagcttgcggatgatcattata 720
            |||||||||| |||||||| |||||||||||| | ||||||||||||||||||||||||
ABV1 : 660  atatgtacagggcgcgtgctgcaaaaaaacaactacaggagcttgcggatgatcattata 719

V95uk: 721  tacattatatacgggttgtcaatactatcttgctgacggtgatgtcttgtaagagcgcaa 780
            |||||||||||||||||||||||||||||| ||||||||||||||||||||| ||||
ABV1 : 720  tacattatatacgggttgtcaatactatcttgttgacggtgatgtcttgtaagagtgcaa 779
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```
V95uk: 781  ttttgccaccagttttgaatttcaatgcgaacgggcctactgggcacgaaacgtatgata 840
            | || |||||||||||||| |||||||||||||| || ||||| |||||||| ||||| |
ABV1 : 780  tattaccaccagttttgaacttcaatgcgaacggacccactggacacgaaacatatgaca 839

V95uk: 841  accattattggggcgagcctcaaaggaacatgaagtttactagtttgccgacggaagcga 900
            | ||||||||||||||||||||||||||||||||||||||||||||| || || |||||||
ABV1 : 840  atcattattggggcgagcctcaaaggaacatgaagtttactagtttaccaacagaagcga 899

V95uk: 901  ttgaaactgggatgctgccatacacatatctggaagagaagagtgatagacaattacagc 960
            |||||||||| ||||||||||||||||||||||||||||||||||||| || |||||||
ABV1 : 900  ttgaaactggcatgctgccatacacatatctggaagagaagagtgataggcagttacagc 959

V95uk: 961  cacaggaagggtgccggatgttgttcgttaccgcggaggaaaacatatatgtgcaaggtg 1020
            |||| ||||| || |||||||||||||||||||||||||||| ||||||||||||||||
ABV1 : 960  cacaagaaggatgtcggatgttgttcgttaccgcggaggaaaatatatatgtgcaaggtg 1019

V95uk: 1021 tcagtaatgatatgccggcagcggatgtaatgtgttgtttgaacaataataacgcgcatg 1080
            |||||||||||||||||||||||||||| |||||||||||||||| || || || ||||
ABV1 : 1020 tcagtaatgatatgccggcagcggatgttatgtgttgtttgaacaacaacaatgcacatg 1079

V95uk: 1081 acatgactatatatgcaatgcggaataaaatgactaacgaatatactatattttacgtgc 1140
            ||||||| |||||||||||||||||||||||| |||||||| ||||||||||||||||
ABV1 : 1080 acatgaccatatatgcaatgcggaataaaatgaccaacgaatacactatattttacgtgc 1139

V95uk: 1141 agactccgggctatgttagtgatgggttgcgcaacaaaataaaaactcagaagaatgtat 1200
            |||||||||| |||||||||||||||||||| |||||||||||||||||| ||||||| |
ABV1 : 1140 agactccgggttatgttagtgatgggttgcgtaacaaaataaaaactcaggagaatgtgt 1199

V95uk: 1201 atcggtgtacagcatttgacgaccagaacgacaatgagttgatggagcatttgcagccaa 1260
            | || |||||||||||||||||||||||||||||| |||||||||||||||| |||||||
ABV1 : 1200 accgatgtacagcatttgacgaccagaacgacaacgagttgatggagcatttacagccaa 1259

V95uk: 1261 cacatttcgctgcgtttatgtatacgccgataggcgcggacggcaagacgatagagaccc 1320
            | ||||| |||||||||||||| |||||||||||||| |||||||||| |||||||||
ABV1 : 1260 cgcattttgctgcgtttatgtacacgccgataggcgcagacggcaagacaatagagacct 1319

V95uk: 1321 tggacggaacgatggtggagttggaagggttggaaccagaaagaacgaaatttataggga 1380
            | |||||||||||||||||||||||||||| |||| || |||||||| ||| |||||||||
ABV1 : 1320 tagacggaacgatggtggagttggaagggctggagcctgaaagaacaaaacttataggga 1379

V95uk: 1381 atgatttcaggaaagatatggcatggatgtcgtttttcgtgtcccgagtgatgtatacag 1440
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
ABV1 : 1380 atgatttcaggaaagatatggcgtggatgtcgtttttcgtgtcccgagtgatgtatacag 1439

V95uk: 1441 gagaaaaggtagtgcataggccgcgtgatagaatcagggtgaaaacttcaatcgctgaac 1500
            |||||||||| ||||| ||||| |||||||| |||||||| ||||||||||||||||||
ABV1 : 1440 gagaaaaggtggtgcacaggccacgtgataggatcagggttaaaacttcaatcgctgaac 1499

V95uk: 1501 atgaatatggtcagggtttagtgagacataagtacaaaaattgcttcaaagatggggtag 1560
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ABV1 : 1500 atgaatatggtcagggtttagtgagacataagtacaaaaattgcttcaaagatggggtag 1559

V95uk: 1561 catcgaagtacagagcgtattcacaaggagtggcagccgtgttaagcgcgctagaggtgg 1620
            |||||||||||| | |||||||||||||||||||||| ||| ||||| ||||||||||||
ABV1 : 1560 catcgaagtaccgggcgtattcacaaggagtggcagctgtgctaagcccgctagaggtgg 1619

V95uk: 1621 aagttaagccaggcaggttagatttaccgttaaatgcaacagtatgtcaagatgtgttgg 1680
            |||||||||||||||||||| |||| || || |||||||| ||||||||||||||||||||
ABV1 : 1620 aagttaagccaggcaggttaaatttgcctttgaatgcaaccgtatgtcaagatgtgttgg 1679

V95uk: 1681 aggtattcatggatatggaatatatgcaatgcaagacaaatggtgaagataggatggtaa 1740
            |||| |||||||||||||||| ||||||||||||||||||||||||||||||||||| |
ABV1 : 1680 aggtgttcatggatatggaatacatgcaatgcaagacaaatggtgaagataggatggtga 1739
```

```
V95uk: 1741 taccgtatgcagtgggcgcgatattatataaaaatggagaagcaatcagcgaagtgtttg 1800
            |||||||||||||| |||||||||| ||||||||||||||||||||||||||||||||||
ABV1 : 1740 taccgtatgcagtaggcgcgatattgtataaaaatggagaagcaatcagcgaagtgtttg 1799

V95uk: 1801 cgagcgtcacgaaagaggacttaataagtggaatgcccaaggtcagatcttatgtagatc 1860
            ||||||| |||||||| ||||| |||||||||||||||||||||||||||||||||| |
ABV1 : 1800 cgagcgttacgaaagaagacttgataagtggaatgcccaaggtcagatcttatgtagacc 1859

V95uk: 1861 caggggtaactgatatgcatgggcaattggaaaaacgtttggccaaaatgaatacagatt 1920
            |||||||||||||||||| || |||||||||||| |||||||||||||||||| || |
ABV1 : 1860 caggggtaactgatatgcacggacaattggaaaaactttggccaaaatgaatacggaat 1919

V95uk: 1921 gggaaagtgaagtgtcgtcaagagatgtgacaaaattgttagaaataataagagaggtaa 1980
               |||||||||| ||| ||||||||||||||||||||||||||||||||||||||||
ABV1 : 1920 cccaaagtgaagtatcgacaagagatgtgacaaaattgttagaaataataagagaggtaa 1979

V95uk: 1981 gtgaaaataatatcaatgtgtatgccaaaggagtgaccaatgataatatagcagtgcatg 2040
            |||||||||||||||||||||||||| || || ||||||||||||||||||||||||
ABV1 : 1980 gtgaaaataatatcaatgtgtatgccaaggggtaaccaatgataatatagcagtgcatg 2039

V95uk: 2041 gagaaataagtgagcggccgacaagtgctctgaccgtgtataagttaggcaaaaatgcta 2100
            |||||||||||||||   | || |||||| |||||||||||||||||||||||| |
ABV1 : 2040 gagaaataagtgagccaacaacgagtgctttgaccgtgtataagttaggcaaaaatgcca 2099

V95uk: 2101 agctgaagaaacgattaggggaagcaggcctaggtgtaggaaaactttctgactatacta 2160
            |||| |||||||||||||||||||| |||||||||| |||||||||| |||||||
ABV1 : 2100 agctaaagaaacgattaggggaagcaggtctaggtgtaggtaaactttctgattatacta 2159

V95uk: 2161 cagaagtggagcaccggccaatacatgaaataaagctgtttgcaagtgcgattgggttta 2220
            |||||||||||||||||||||||||||||||||  | || ||||||||||||||||||
ABV1 : 2160 cagaagtggagcaccggccaatacatgaaataaaattattcgcaagtgcgattgggttta 2219

V95uk: 2221 aaagttatgatgagccggaaagaatgaatgaatcgatgtttgaggagaagatcaatgagg 2280
            |||||||||||||||||||||||||||||||||||||||||  |||||||||||||| |
ABV1 : 2220 aaagttatgatgagccggaaagaatgaatgaatcgatgtttgctgagaagatcaatgacg 2279

V95uk: 2281 tgataagtggatataagtgagcataaaggtatgtaaattactatagatacgccgaacacg 2340
            |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
ABV1 : 2280 tgataagtggatataagtgagcataaaggtatgtaaatcactatagatacgccgaacacg 2339

V95uk: 2341 atcagtcaataactatgaataagttaatgac 2371
            |||||||||||||||||||||||||||||
ABV1 : 2340 atcagtcaataactatgaataagttaatgac 2370
```

Fig.III.3 The nucleotide sequence of 2371 nt of dsRNA L5 *A. bisporus* isolate V95 from The UK compared with dsRNA L5 *A. bisporus* (ABV1) from The Netherlands.

Appendix III-4

X94362) L5 [Agaricus bisporus virus 1] Length = 724,
Score = 1411 bits (3612), Expect = 0.0, Identities = 706/722 (97%),
Positives = 713/722 (97%) Frame = +3

```
V95uk: 126  MTTQQASVMRFTTAYSSNNKDANDVVPGSLLHQRLVKSRELEEHVTRNYGLPDVMYSYAG 305
            MTTQQASVMRFTTAYSSNNK+ANDVVPGSLLHQRLVKSRELEEHVTRNYGLPDVMYSYAG
ABV1 : 1    MTTQQASVMRFTTAYSSNNKNANDVVPGSLLHQRLVKSRELEEHVTRNYGLPDVMYSYAG 60

V95uk: 306  TLPGEYARIGSQIEKQIIGKNLSNILLELCDGDNMERVKMVTLCHDVMHNKVQKIAASRT 485
            TLPGEYARIGSQIEKQIIGKNLSNILLELCDGDNMERVKMVTLCHDVMHNKVQKIAASRT
ABV1 : 61   TLPGEYARIGSQIEKQIIGKNLSNILLELCDGDNMERVKMVTLCHDVMHNKVQKIAASRT 120

V95uk: 486  SCKFEVSEQSDMEVKIEVAFGSAEDGYQTKELVITFEKVNDQWAFVGMEALNDFRKVRNM 665
            SCKFEVSEQSDMEVKIEVAFGS EDG QTKELVITFEKVNDQWAFVGMEALNDFRKVRNM
ABV1 : 121  SCKFEVSEQSDMEVKIEVAFGSPEDGCQTKELVITFEKVNDQWAFVGMEALNDFRKVRNM 180

V95uk: 666  YRARAAKKQLQELADDHYIHYIRVVNTILLTVMSCKSAILPPVLNFNANGPTGHETYDNH 845
            YRARAAKKQLQELADDHYIHYIRVVNTILLTVMSCKSAILPPVLNFNANGPTGHETYDNH
ABV1 : 181  YRARAAKKQLQELADDHYIHYIRVVNTILLTVMSCKSAILPPVLNFNANGPTGHETYDNH 240

V95uk: 846  YWGEPQRNMKFTSLPTEAIETGMLPYTYLEEKSDRQLQPQEGCRMLFVTAEENIYVQGVS 1025
            YWGEPQRNMKFTSLPTEAIETGMLPYTYLEEKSDRQLQPQEGCRMLFVTAEENIYVQGVS
ABV1 : 241  YWGEPQRNMKFTSLPTEAIETGMLPYTYLEEKSDRQLQPQEGCRMLFVTAEENIYVQGVS 300

V95uk: 1026 NDMPAADVMCCLNNNNAHDMTIYAMRNKMTNEYTIFYVQTPGYVSDGLRNKIKTQKNVYR 1205
            NDMPAADVMCCLNNNNAHDMTIYAMRNKMTNEYTIFYVQTPGYVSDGLRNKIKTQ+NVYR
ABV1 : 301  NDMPAADVMCCLNNNNAHDMTIYAMRNKMTNEYTIFYVQTPGYVSDGLRNKIKTQENVYR 360

V95uk: 1206 CTAFDDQNDNELMEHLQPTHFAAFMYTPIGADGKTIETLDGTMVELEGLEPERTKFIGND 1385
            CTAFDDQNDNELMEHLQPTHFAAFMYTPIGADGKTIETLDGTMVELEGLEPERTK IGND
ABV1 : 361  CTAFDDQNDNELMEHLQPTHFAAFMYTPIGADGKTIETLDGTMVELEGLEPERTKLIGND 420

V95uk: 1386 FRKDMAWMSFFVSRVMYTGEKVVHRPRDRIRVKTSIAEHEYGQGLVRHKYKNCFKDGVAS 1565
            FRKDMAWMSFFVSRVMYTGEKVVHRPRDRIRVKTSIAEHEYGQGLVRHKYKNCFKDGVAS
ABV1 : 421  FRKDMAWMSFFVSRVMYTGEKVVHRPRDRIRVKTSIAEHEYGQGLVRHKYKNCFKDGVAS 480

V95uk: 1566 KYRAYSQGVAAVLSALEVEVKPGRLDLPLNATVCQDVLEVFMDMEYMQCKTNGEDRMVIP 1745
            KYRAYSQGVAAVLS LEVEVKPGRL+LPLNATVCQDVLEVFMDMEYMQCKTNGEDRMVIP
ABV1 : 481  KYRAYSQGVAAVLSPLEVEVKPGRLNLPLNATVCQDVLEVFMDMEYMQCKTNGEDRMVIP 540

V95uk: 1746 YAVGAILYKNGEAISEVFASVTKEDLISGMPKVRSYVDPGVTDMHGQLEKRLAKMNTDWE 1925
            YAVGAILYKNGEAISEVFASVTKEDLISGMPKVRSYVDPGVTDMHGQLEK LAKMNT+ +
ABV1 : 541  YAVGAILYKNGEAISEVFASVTKEDLISGMPKVRSYVDPGVTDMHGQLEKLLAKMNTESQ 600

V95uk: 1926 SEVSSRDVTKLLEIIREVSENNINVYAKGVTNDNIAVHGEISERPTSALTVYKLGKNAKL 2105
            SEVS+RDVTKLLEIIREVSENNINVYAKGVTNDNIAVHGEISE  TSALTVYKLGKNAKL
ABV1 : 601  SEVSTRDVTKLLEIIREVSENNINVYAKGVTNDNIAVHGEISEPTTSALTVYKLGKNAKL 660


V95uk: 2106 KKRLGEAGLGVGKLSDYTTEVEHRPIHEIKLFASAIGFKSYDEPERMNESMFEEKINEVI 2285
            KKRLGEAGLGVGKLSDYTTEVEHRPIHEIKLFASAIGFKSYDEPERMNESMF EKIN+VI
ABV1 : 661  KKRLGEAGLGVGKLSDYTTEVEHRPIHEIKLFASAIGFKSYDEPERMNESMFAEKINDVI 720

V95uk: 2286 SG 2291
            SG
ABV1 : 721  SG 722
```

Fig.III.4 The amino acid sequence of dsRNA L5 *A. bisporus* isolate V95 from The UK compared with dsRNA L5 *A. bisporus* (ABV1) from The Netherlands.

Appendix III-5

DsRNA L5 *Agaricus bisporus*

ref|NP_003476.1| G protein-coupled receptor 68
>gi|3024266|sp|Q15743|GP68_HUMAN
PUTATIVE G PROTEIN-COUPLED RECEPTOR GPR68 (OVARIAN CANCER
G PROTEIN-COUPLED RECEPTOR 1) (OGR-1)
>gi|1457939|gb|AAC50596.1| (U48405) G protein coupled
receptor OGR1 [Homo sapiens]
Length = 365

Score = 35.2 bits (79), Expect = 2.4
Identities = 31/109 (28%), Positives = 54/109 (49%), Gaps = 4/109 (3%)
Frame = -3

```
V95uk: 1376 YKFRSFWFQPFQLHHRSVQGLYRLAVRAYRRIH--KRSEMCWLQMLHQLIVVLVVKCCTP 1203
            Y+F   +  P  L   S QG+    +RA RR H  ++S    +Q L    VV+ + C  P
Sbjct: 188  YRFLVGFLFPICLLLASYQGI----LRAVRRSHGTQKSRKDQIQRLVLSTVVIFLACFLP 243

V95uk: 1202 IYILLSFYFVAQPITNIARSLHVKY--SIFVSHFIPHCIYSHVMRVIIVQTTH 1050
             ++LL    V +   + A+ +   Y  S+ ++ F  +C+   V+   + +TTH
Sbjct: 244  YHVLLLVRSVWEASCDFAKGVFNAYHFSLLLTSF--NCVADPVLYCFVSETTH 294
```

gb|AAB94475.1 (AF003534)putative global transactivator [Chilo iridescent virus]
Length = 606

Score = 34.8 bits (78), Expect = 3.2
Identities = 19/65 (29%), Positives = 33/65 (50%)
Frame = +3

```
V95uk: 579 LVITFEKVNDQWAFVGMEALNDFRKVRNMYRARAAKKQLQELADDHYIHYIRVVNTILLT 758
           LVIT + V  +W   G+E   D   +R +Y  R   K + +++ D  + Y  V+ T  +
Sbjct: 96  LVITSKTVMHEWKTEGVEKFFDSDNIRVLYLHRDYIKNIDKISRDDIMTYDIVITTYDVC 155

V95uk: 759 VMSCK 773
           + +CK
Sbjct: 156 LFACK 160
```

gb|AAB33905.1|(S75674)DNA helicase homolog [Chilo iridescent virus CIV, insect iridescent virus type 6, Peptide, 606 aa]

>gi|555221|**gb|AAA62412.1**| (M81388) helicase [Chilo iridescent virus]
Length = 606

Score = 34.8 bits (78), Expect = 3.2
Identities = 18/65 (27%), Positives = 34/65 (51%)
Frame = +3

```
V95uk: 579 LVITFEKVNDQWAFVGMEALNDFRKVRNMYRARAAKKQLQELADDHYIHYIRVVNTILLT 758
          LVIT + V  +W   G+E   D   +R +Y  ++  K + +++ D  + Y  V+ T  +
Sbjct: 96  LVITSKTVMHEWKTEGVEKFFDSDNIRVLYLHKSYIKNIDKISRDDIMTYDIVITTYDVC 155

V95uk: 759 VMSCK 773
          + +CK
Sbjct: 156 LFACK 160
```

gb|AAC05611.1| (U88366) orphan G protein-coupled receptor bRGR1 [Bos taurus]
>gi|2827878|gb|AAC05612.1| (U88367) orphan G protein-coupled receptor bRGR1b [Bos taurus]
Length = 361

Score = 34.0 bits (76), Expect = 5.4
Identities = 25/88 (28%), Positives = 46/88 (51%), Gaps = 4/88 (4%)
Frame = -3

```
V95uk: 1313 YRLAVRAYRRIH--KRSEMCWLQMLHQLIVVLVVKCCTPIYILLSFYFVAQPITNIARSL 1140
           YR  +RA RR H  ++S    +Q L    VV+ + C  P ++LL    + +   + A+ +
Sbjct: 205  YRGILRAVRRSHGTQKSRKDQIQRLVLSTVVIFLACFLPYHVLLLVRSLWESSCDFAKGI 264

V95uk: 1139 HVKY--SIFVSHFIPHCIYSHVMRVIIVQTTH 1050
              Y  S+ ++ F  +C+   V+   + +TTH
Sbjct: 265  FNAYHFSLLLTSF--NCVADPVLYCFVSETTH 294
```

Fig. III.5 A portion of alignment of the putative polypeptide of dsRNA L5 in *A. bisporus* isolate V95 and published polypeptide sequence using BLAST sequence similarity searching method (Altschul *et al.*, 1997).

Appendix III-6

DsRNA 1 *Verticillium fungicola*

sp|P36286|POLN_SMSV1 NON-STRUCTURAL POLYPROTEIN [CONTAINS: RNA-DIRECTED RNA POLYMERASE ;THIOL PROTEASE ; HELICASE (2C LIKE PROTEIN)] Length = 1156

Score = 35.2 bits (79), Expect = 0.21
Identities = 32/128 (25%), Positives = 56/128 (43%), Gaps = 4/128 (3%)
Frame = +1

```
V7-3 : 535  CRPRTRLMGQAL----ALGVGMLGSSLDKVARHLGKRGVSLLDCDAVMSDIAKWDANMPE 702
            CR   + + +++    ALG   +G ++D  A     + +     D    D +KWD+  P
Sbjct: 825  CRAAFKRVSESIMANHALGFIQVGINMDGPAVEDPFKRLERPKHDRYCVDYSKWDSTQPP 884

V7-3 : 703  VLIAAAFDLMESVVDKSGLDAVGRATRSLMVDVAKRQLMVKLIEHPAGYFLELFGCMPSG 882
             + + + D++    DKS +           VD A   L    I    G   ++ G +PSG
Sbjct: 885  KVTSQSIDILRHFTDKSPI----------VDSACATLKSNPIGIFNGVAFKVAGGLPSG 933

V7-3 : 883  SFYTSCINTIGN 918
            TS IN++ +
Sbjct: 934  MPLTSIINSLNH 945
```

gb|AAC57049.1| (U52093) RNA dependent RNA polymerase 3D [San Miguel sea lion virus] Length = 150

Score = 32.8 bits (73), Expect = 4.6
Identities = 25/88 (28%), Positives = 40/88 (45%), Gaps = 4/88 (4%)
Frame = +1

```
V7-3 : 655 DAVMSDIAKWDANMPEVLIAAAFDLMESVVDKSGLDAVGRATRSLMVDVAKRQLMVKLIE 834
           D    D +KWD+  P  + + + D+     DKS +           VD A      +L
Sbjct: 18  DRYCVDYSKWDSTQPPKVTSQSIDIHRHFTDKSPI----------VDSA----CARLKS 62

V7-3 : 835 HPAGYF----LELFGCMPSGSFYTSCINTIGN 918
           +P G F     ++ G +PSG   TS IN++ +
Sbjct: 63  NPVGIFNGVAFKVAGGLPSGMPLTSIINSLNH 94
```

dbj|BAA34783.1| (AB012616) RNA-dependent RNA polymerase [Pyrus pyrifolia] Length = 477

Score = 33.2 bits (74), Expect = 3.5
Identities = 22/83 (26%), Positives = 41/83 (48%)
Frame = +1

```
V7-3 : 670 DIAKWDANMPEVLIAAAFDLMESVVDKSGLDAVGRATRSLMVDVAKRQLMVKLIEHPAGY 849
           D  ++DA +    I AAFD++  +++             +  +++++  + K I  P GY
Sbjct: 255 DWKQFDATVSRFEINAAFDIIMDLIEFPNYPTY------VAFELSRQLFIHKKIAAPDGY 308

V7-3 : 850 FLELFGCMPSGSFYTSCINTIGN 918
                +PSGS++TS I +I N
Sbjct: 309 IYWSHKGIPSGSYFTSIIGSIIN 331
```

dbj|BAA74537.1| (AB022887) polyprotein [navel orange infectious mottling virus]
Length = 872

Score = 32.8 bits (73), Expect = 4.6
Identities = 24/84 (28%), Positives = 40/84 (47%)
Frame = +1

```
V7-3 : 670 DIAKWDANMPEVLIAAAFDLMESVVDKSGLDAVGRATRSLMVDVAKRQLMVKLIEHPAGY 849
          D + +D + P +L+ A  D +  +   S ++   R  R+++  V  R L+        GY
Sbjct: 475 DYSGFDTSTPRILVYAIVDKINELAGDSEMNQ--RTRRNIIRFVLNRYLISD------GY 526

V7-3 : 850 FLELFGCMPSGSFYTSCINTIGND 921
            E+ G  PSG   T  IN+I N+
Sbjct: 527 VYEIHGGTPSGFAPTVMINSIVNE 550
```

sp|P13887|POLN_RRVN virus NONSTRUCTURAL POLYPROTEIN [CONTAINS: NONSTRUCTURAL PROTEINS
NSP1 to NSP4] Length = 2479

Score = 30.1 bits (66), Expect = 6.9
Identities = 27/88 (30%), Positives = 40/88 (44%), Gaps = 2/88 (2%)
Frame = +1

```
V7-3 : 655  DAVM-SDIAKWDANMPEVLIAAAFDLMESV-VDKSGLDAVGRATRSLMVDVAKRQLMVKL 828
           DAV+ +DIA +D +  + L   A  L+E + VD+  LD +  A              +
Sbjct: 2233 DAVLETDIASFDKSQDDSLALTALMLLEDLGVDQELLDLIEEAFGE----------ITS 2281

V7-3 : 829  IEHPAGYFLELFGCMPSGSFYTSCINTIGN 918
           +  P G   +   M SG F T  INT+ N
Sbjct: 2282 VHLPTGTRFKFGAMMKSGMFLTLFINTLLN 2311
```

Fig.III.6 A portion of alignment of the putative polypeptide of dsRNA 1 in *V. fungicola* isolate V7-3 and published polypeptide sequence using BLAST sequence similarity searching method (Altschul *et al.*, 1997).

Appendix III-7

DsRNA 2 *Trichoderma harzianum*

sp|P20131|V70K_TYMVA 69 KD PROTEIN : turnip yellow mosaic virus
Length = 628

Score = 30.5 bits (67), Expect = 4.5
Identities = 23/65 (35%), Positives = 30/65 (45%), Gaps = 5/65 (7%)

```
T7   : 139 LREFTRQPSFPLPMETRITTGMETSDHPIHEPLDVEPTNTAPVTPVLRTSVKSME-TTFD 197
          LR+  R PSFP P +T           P   P D+ P ++ PV  V RT V + E  TF
Sbjct: 453 LRDLGRTPSFPTPPKTSTRAPESCIAAP---PTDIAPLDSDPVLSV-RTEVHAPERRTFM 508

T7   : 198 SPPVI 202
           P  +
Sbjct: 509 DPEAL 513
```

sp|P38681|NIR_NEUCR NITRITE REDUCTASE [NAD(P)H] >gi|542387|pir||A49848 nitrite reductase - Neurospora crassa
Length = 1176

Score = 32.8 bits (73), Expect = 4.2
Identities = 21/70 (30%), Positives = 33/70 (47%), Gaps = 1/70 (1%)
Frame = -3

```
T7   : 629 ELTVVETAVALGSEDVGITGGESKVIDLTEVRRT-GVTGAVLVGSTSRGSCIG*SDIPVV 453
          EL V  + + LG++  G   G+  + D T++     VT A LV     G C    D+
Sbjct: 465 ELDVPPSQLILGAKKSGDDNGDDDLPDDTQICSCHNVTKADLVAPLKSGECTSLGDLKSC 524

T7   : 452 IRMGSGKDGCL 420
           + G+G  GC+
Sbjct: 525 TKAGTGCGGCM 535
```

Fig.III.7 A portion of alignment of the putative polypeptide of dsRNA 2 of *T. harzianum* isolate T7 and published polypeptide sequence using BLAST sequence similarity searching method (Altschul *et al.*, 1997).

Appendix III-8

DsRNA 3 *Trichoderma harzianum*

emb|CAA73808.1| (Y13381) amphiphysin [Rattus norvegicus]
Length = 683

Score = 36.4 bits (82), Expect = 0.33
Identities = 37/148 (25%), Positives = 63/148 (42%), Gaps = 7/148 (4%)
Frame = +1

```
T7   : 1   FSYASRKKLSSFWPSSSMMFYVVHLMNARLVDHFYFKRYCPSYHPYILRLYFAIL-FYIQ 177
           F+   +++L S W S  + FYV    N   ++  + K      H    +LY  +     Q
Sbjct: 183 FNVDLQEELPSLW-SRRVGFYVNTFKNVSSLEAKFHKEIAVLCH----KLYEVMTKLGDQ 237

T7   : 178 CLRAASDVNALPEDQHQFLIRFLQAHPSESLPVPGPLLTLFKSICTS-----QPEIQSYG 342
              A  +   P D     I    + P E+ P+P P  +   ++  +     +P   S
Sbjct: 238 HADKAFSIQGAPSDSGPLRIAKTPSPPEEASPLPSPTASPNHTLAPASPAPVRPRSPSQT 297

T7   : 343 KIYPRIPPSPGPERRSEFRLDNSVSFFQPN-VPGI 444
           +  P +PP P      E + +N ++FF+ N VP I
Sbjct: 298 RKGPPVPPLPKVTPTKELQQENIINFFEDNFVPEI 332
```

gb|AAC02977.1| (AF034996) amphiphysin I [Homo sapiens]
Length = 653

Score = 36.4 bits (82), Expect = 0.33
Identities = 38/148 (25%), Positives = 62/148 (41%), Gaps = 7/148 (4%)
Frame = +1

```
T7   : 1   FSYASRKKLSSFWPSSSMMFYVVHLMNARLVDHFYFKRYCPSYHPYILRLYFAIL-FYIQ 177
           F+   +++L S W S  + FYV    N   ++  + K      H    +LY  +     Q
Sbjct: 183 FNVDLQEELPSLW-SRRVGFYVNTFKNVSSLEAKFHKEIAVLCH----KLYEVMTKLGDQ 237

T7   : 178 CLRAASDVNALPEDQHQFLIRFLQAHPSESLPVPGPLLTLFKSICTS-----QPEIQSYG 342
              A  +   P D     I    + P E  P+P P  +   ++  +     +P   S
Sbjct: 238 HADKAFTIQGAPSDSGPLRIAKTPSPPEEPSPLPSPTASPNHTLAPASPAPARPRSPSQT 297

T7   : 343 KIYPRIPPSPGPERRSEFRLDNSVSFFQPN-VPGI 444
           +  P +PP P      E + +N +SFF+ N VP I
Sbjct: 298 RKGPPVPPLPKVTPTKELQQENIISFFEDNFVPEI 332
```

gb|AAC34351.1| (AC004260) Putative nuclear protein [Arabidopsis thaliana]
Length = 613

Score = 32.1 bits (71), Expect = 6.3
Identities = 27/90 (30%), Positives = 44/90 (48%), Gaps = 21/90 (23%)
Frame = +1

```
T7   : 487 NPIYPPKGKHIPVTATATNPEVFGFHTFPVPTDRSDAEKWSL--------NSAGLEFPCE 642
           +P+ PPK KH  V   + +P V   H+ P P    D + W +        N  G   P +
Sbjct: 217 DPLDPPKFKHKRVPRASGSPPVPVMHSPPRPVTVKDQQDWKIPPCISNWKNPKGYTIPLD 276

T7   : 643 ----------ADSKLNESFAERYEDFDFPTMTANDDL---SSITNYLGMKD 756
                     D ++N++FA+  E        A + +   S +   + MKD
Sbjct: 277 KRLAADGRGLQDVQINDNFAKLSEALYVAEQKAREAVSMRSKVQKEMVMKD 327
```

sp|P87034|GBA3_USTMA GUANINE NUCLEOTIDE-BINDING PROTEIN ALPHA-3 SUBUNIT
>gi|1930023|gb|AAC49726.1| (U85777) G protein alpha subunit [Ustilago maydis]
Length = 354

Score = 32.5 bits (72), Expect = 4.8
Identities = 22/76 (28%), Positives = 32/76 (41%)
Frame = +1

```
T7   : 379 ERRSEFRLDNSVSFFQPNVPGIFALLTHLDGVINGANPIYPPKGKHIPVTATATNPEVFG 558
           ER SEF L +S ++F  NV  I          +   N +   + K   ++ T  N
Sbjct: 143 ERSSEFYLMDSAAYFFDNVNRIGQ-----SDYVPNENDVLRARSKTTGISETRFNMGQLS 197

T7   : 559 FHTFPVPTDRSDAEKW 606
            H F V   RS+ +KW
Sbjct: 198 IHLFDVGGQRSERKKW 213
```

sp|O14438|GBA3_USTHO GUANINE NUCLEOTIDE-BINDING PROTEIN ALPHA-3 SUBUNIT
>gi|2231316|gb|AAC49880.1| (U76672) Fil1 [Ustilago hordei]
Length = 354

Score = 31.7 bits (70), Expect = 8.3
Identities = 22/76 (28%), Positives = 32/76 (41%)
Frame = +1

```
T7   : 379 ERRSEFRLDNSVSFFQPNVPGIFALLTHLDGVINGANPIYPPKGKHIPVTATATNPEVFG 558
           ER SEF L +S ++F  NV  I          +   N +   + K   ++ T  N
Sbjct: 143 ERSSEFYLMDSAAYFFDNVNRIGQA-----DYVPDENDVLRARSKTTGISETRFNMGQLS 197

T7   : 559 FHTFPVPTDRSDAEKW 606
            H F V   RS+ +KW
Sbjct: 198 IHLFDVGGQRSERKKW 213
```

Fig.III.8 A portion of alignment of the putative polypeptide of dsRNA 3 in *T. harzianum* isolate T7 and published polypeptide sequence using BLAST sequence similarity searching method (Altschul *et al.*, 1997).